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Final Report
2.2-17

Dietary Sulphate, Copper Induced Thiamine
Deficiency in Livestock

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Final Report
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Abstract

It is well established that some Saskatchewan livestock water supplies are high in sulphate content. Previous research has demonstrated an effect of these high sulfate waters on copper status and copper utilization by cattle. The current studies were carried out to determine the effect of these high sulfate waters on thiamine utilization and additional interactions involving nutrients such as thiamine when cattle are exposed to these high sulfate waters. It was established early in the project that cattle are adversely affected by high sulfate intake through an effect on thiamine metabolism. This adverse effect could be duplicated through exposure of sheep under laboratory conditions to high levels of sulfur intake. As part of this research, it was discovered that traditional thiamine assays were not entirely satisfactory and a modified method using protozoa was developed and verified as an improved method. As part of this work it was also discovered that in addition to the effect of sulfate on thiamine status, that exposure to lead depressed blood levels and that repeated injections of thiamine decreased the lead content in bone and brain but increased the lead content in organs involved in excretion of lead such as the kidney. These studies have demonstrated, that although very little can be done economically to remove sulfate from Saskatchewan waters, a number of management

procedures can be implemented to alleviate the effects of high sulfur intake.

The reviewers asked that specific management procedures be identified in the abstract.

These management procedures include:

- 1) The addition of copper to the ration at levels of 20-50 mg/kg of ration depending on the sulfate level in the water.
- 2) The addition of 100-300 mg of thiamine to the ration of mature cattle daily. This addition of thiamine would only take place where there has been some evidence of polioencephalomalacia.
- 3) In some situations it may be practical to collect surface water in dugouts to use in place of high sulfate water from wells or to blend with water from a high sulfate well to reduce the total concentration of sulfate in the water consumed by livestock to less than 2,000 mg/l.

Summary of Research and Impact on Saskatchewan Agriculture

The Agricultural Development Fund has supported a major research program in the Department of Animal and Poultry Science, from 1980 through 1988. This research began in 1973 with the observations in the Yorkton area that cattle were suffering from a copper deficiency. Surveys were conducted in cooperation with the Soil Science department and the Saskatchewan Feed Testing Laboratory which verified that soil and feeds were marginal in copper content. Saskatchewan Research Council records (Rutherford) were reviewed which indicated the high sulfate content of many Saskatchewan waters used by livestock. This high sulfur content appeared to be mainly in wells more than 20 meters in depth. M.Sc. studies conducted by R. Ford, K. Bergon and the Ph.D. program of M.E. Smart provided further background to the copper deficiency situation found in Saskatchewan. Dr. Smart has continued her interest and has conducted an effective extension program on methods for preventing or alleviating copper deficiency in cattle. As a result of these studies, which were concluded by about 1980, it was recognized that there were many deficiencies in our basic understanding of copper metabolism in cattle and the interaction of sulfur and other nutrients in this complex deficiency syndrome. In 1981, a world wide search was undertaken to identify and hire with ADF funding a world class mineral metabolism researcher.

Dr. R. Gooneratne was hired and played a lead role in the Department of Animal and Poultry Science mineral research program between November of 1982 and October of 1988 when he was appointed to the faculty of Lincoln College, Christchurch, New Zealand. The 59 publications arising from this research have provided a broad understanding of the copper/sulfur and related interactions. In this work, Dr. Gooneratne cooperated effectively with other members of this department, with faculty of the Western College of Veterinary Medicine, and with Saskatchewan Department of Agriculture staff.

The research reported in this final report highlights the findings of the past several years. A problem involving low copper intake, high sulfate intake and a thiamine deficiency was observed initially in a cow calf farm operation in south east Saskatchewan. A condition described as polioencephalomalacia (PEM) was identified. A relationship between PEM and high levels of sulfur intake had not previously been studied in cattle in Canada, although some information was available on a suspected role of high sulfur intake in this condition. Cattle injected with thiamine on this farm responded dramatically in improved health, appearance, and performance. In a subsequent experiment with sheep at the University of Saskatchewan, it was shown that PEM could be experimentally produced in sheep given high levels of sulfur in the diet. Brain lesions

consistent with a thiamine deficiency (Vitamin B₁) were produced in this work. It was suspected that a high sulfur intake could lead to impaired performance of cattle through alteration in brain function leaving to changes in feeding behavior and ability to respond to environmental stimuli. Specific lesions in certain areas of the brain were identified and an impairment in hearing was observed using carefully controlled laboratory conditions. This hearing impairment could be prevented through supplementation with thiamine.

In establishment of a thiamine assay, a review of literature of the available methods was conducted and several methods were established for testing of pure thiamine and thiamine in known concentrations from a variety of sources. One of the most sensitive and effective methods used a bacteria (*Lactobacillus fermenti*). In testing this organism as in a thiamine assay, it was discovered that it responded to portions of the thiamine molecule which are not metabolically active in cattle and other mammals. The use of this assay, therefore, would overestimate the effective thiamine level in the diet. Further review of literature indicated that a protozoa (*Ochromonas danica*) is more accurate in identifying thiamine forms which are active in cattle. This protozoa method was modified to be conducted in large numbers using equipment available for blood testing in the Saskatchewan Research Council blood typing laboratory.

The modifications and automation of this method will be published and undoubtedly adopted world wide as an improved method for thiamine analysis.

In addition to the impaired thiamine status which can result from high sulfur intake, consideration was given to implications of low thiamine status apart from the obvious ones associated with a marginal or deficient state. A review of the literature indicated that thiamine may protect against toxicity of heavy metals such as lead. The question then arose as to the possibility of accumulation of heavy metals in Saskatchewan cattle as a consequence of low thiamine status. Although it was not possible to complete this work within the research program schedule, some progress was made in establishing that thiamine is effective in reducing the amount of lead stored in ruminants. It was also observed that thiamine may not be sufficiently active to be useful where an acute toxicity of lead is encountered.

The reviewers also asked that what should occur in the cattle industry as a result of this project be included in the summary. The added information is as follows:

This project has demonstrated the detrimental effects of high sulfate in drinking water of cattle on utilization of copper and on the effect that low thiamine status may have on brain function. Commercial copper supplements are now available and their use should be encouraged in situations

where high sulfate water is encountered. These recommendations can be made through feed company nutritionists, the Feed Testing Laboratory, Provincial and private livestock advisors and consultants and veterinarians. The use of thiamine in combination with supplementary copper must be considered in some situations. Most veterinarians in Saskatchewan are now aware of the potential therapeutic and preventive value of thiamine in high sulfate water situations.

Although there is some interaction between thiamine and lead, thiamine alone cannot be used in preventing or treating a lead toxicity.

Further research is required to identify situations in which thiamine deficiency may occur. Further research involving histological examination and evaluation by a pathologist is required to identify the significance of lesions which occur during both marginal and severe thiamine deficiency.

Two areas of research associated with high sulfate water remain which should be addressed through further field studies and research. These are evaluation of the effect of high sulfate waters on selenium status and utilization and on zinc status and utilization. There is reason to suspect that the levels of sulfate in cattle drinking water in Saskatchewan could have a detrimental effect on the utilization of these two trace minerals.

Technical Reports

Background: High-Sulfur Related Thiamine Deficiency in Cattle: A Field Study

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Abstract:

Following development of polioencephalomalacia in one of 105 cattle in a farm in south east Saskatchewan, a study was initiated to monitor thiamine (B_1) and copper (Cu) status, and to evaluate interactive nutritional factors which may have been responsible for this occurrence. It was evident that a combination of high sulfur (S) and low Cu intake was responsible for the depletion of blood B_1 and plasma Cu concentration. Supplementation with trace minerals alone resulted in a significant ($P < 0.05$) improvement in both B_1 and Cu status of the herd. We recommend that herds exposed to high intakes of S be supplemented with Cu up to 50 mg/kg feed dry matter to alleviate potential deficiencies of B_1 and Cu.

Impact and Significance for Saskatchewan Agriculture

The high intake of sulfur on some Saskatchewan farms as a result of consumption of high sulfate water may lead to a thiamine deficiency. This deficiency can be alleviated by increasing the proper intake which ties up sulfur and reduces sulfur impairment of thiamine utilization. The condition can also be alleviated by injection of thiamine. This research shows that the performance of cattle ingesting high sulfate waters may be impaired through a number effects and that supplementation with proper may not always be effective.

Introduction

Ruminant species are not considered to require thiamine (B_1) in their diet (1) since sufficient amounts are synthesized in the rumen to meet the demands of the mature animal (2). However, acute (3) and subclinical (4) B_1 deficiencies have been reported. There is little doubt that progressive B_1 deficiency is responsible for field cases of polioencephalomalacia (PEM) which occur in ruminants (1). More recent reports have suggested a possible causal relationship between high intakes of sulfur (S) and PEM (5, 6). Such incidents may occur during long-term consumption of high levels of S, and in those instances the whole herd is potentially at risk. Although, in some cases further intake of excessive amounts of S can be reduced, under practical farming conditions this is not always possible. This is especially so when water contains a high content of S since the cost of water purification is exorbitant. Ruminant livestock in the Canadian Prairies can therefore be regarded as potentially at risk to PEM since water from deep aquifers on many farms contains high levels of S as sulfates (7, 8).

Relevance of PEM induced by high levels of S in cattle in Canada is not known, or is it known whether diets deficient in trace minerals increase the risk of the disease. Adverse effects of high intakes of S on metabolism of copper (Cu) in cattle have been reviewed (7). Although the role of S in decreasing the availability of dietary Cu in sheep is well established (9), the mechanisms underlying S-induced PEM are poorly understood.

The suggested role of S in the induction of both Cu (7) and B_1 (10) deficiency in cattle led us to suggest the existence of a Cu-S- B_1 interaction. In the rumen, S compounds are reduced to sulfides, and these combine readily with Cu to form insoluble Cu sulfides (9). Therefore we believed that in

cattle fed moderate to high levels of S, a diet high in Cu alone might be beneficial in reducing the S load in the rumen. If our hypothesis is correct a high concentration of Cu in the diet alone could be expected to improve indirectly the B₁ status of the herd. This paper reports a field study carried out in south east Saskatchewan to test this hypothesis in cattle at a feedlot with a prior history of PEM. In this study we fed a custom made trace-mineral supplement to animals of low to marginal B₁ and Cu status, and measured its impact on concentrations of B₁ and Cu in blood and plasma respectively.

History

The farm involved was a 105 beef-cattle unit located in south east Saskatchewan. Calves of mixed breeding had been purchased through a local auction market in October 1986. They were separated into two weight classes and housed in two feedlot pens designated as North and South pens.

North pen: Housed 53 heavier calves. Their estimated ages at the time of purchase ranged from nine to eleven months of age and their mean body weight was 340 kg. Many calves in this pen were medium to large frame type and 44% were Simmental-breed crosses.

South pen: Housed the remaining 52 smaller calves. Their estimated ages at the time of purchase ranged from seven to nine months and their mean body weight was 260 kg. Only 18% of the animals in this pen were Simmental crosses.

Feeding and Management: All animals were fed a mixture of forage, barley and canola meal. Based on farmer's records the approximate daily feed intake by

steers in the north pen was 2.72 kg forage, 4.1 kg barley and 0.9 kg canola meal per animal. Feed analysis showed this diet to provide approximately 12.85% protein and 2.18 M cal/kg metabolizable energy. This was within National Academy of Sciences - National Research Council (NAS-NRC) (2) recommendations for this type of cattle. Smaller steers in the south pen were offered approximately 10% less feed than those in the north pen. Trace mineral and S analysis of the feed and water are shown in Table 1. The animals were also offered a free-choice commercial mineral (macro and micro) supplement. The mineral supplement had not been analyzed previously but according to the manufacturer's guaranteed analysis it should have supplied adequate amounts of both macro and microminerals (Table 2). Calves in both pens received water from a 40 m well drilled approximately two years previously. The water from this well had not been analyzed previously. Although the calves were consuming all the feed given to them, the owner had noticed that the animals were somewhat depressed and not growing at the expected rate.

Clinical Findings

On February 4, 1987, the owner noticed that one animal (Simmental mix breed) in north pen was scouring and walking with difficulty and sought veterinary advice (GAK). Examination of this steer (approximately 400 kg body wt) revealed a normal body temperature, passage of loose feces, and associated abdominal pain. The animal was treated with sulfamethazine (Spanbolets, Nordern Laboratories, Lincoln, Nebraska), sodium sulfosuccinate (Dioctol, Rogar/STB Inc, Montreal, Quebec) orally, and oxytetracycline (Liquamycin LP, Rogar/STB Inc, Montreal, Quebec) intravenously (IV) for coccidiosis, colic,

and thromboembolic meningoencephalitis, respectively. The following day the animal was found recumbent and the veterinarian (GAK) was called again. The steer lay in lateral recumbency with legs in an extended position. There was opisthotonus and the animal was shivering. Examination revealed a normal temperature, rigidity of limbs, and loss of vision. At this time a tentative diagnosis of PEM was made and the steer was given B₁ hydrochloride (400 mg) (thiamine hydrochloride, MTC Pharmaceuticals, Cambridge, Ontario) and oxytetracycline (Liquamycin LP, Rogar/STB Inc, Montreal, Quebec) IV. By evening the animal showed some improvement but was unable to get up. The following morning the animal was able to rise and had regained coordination but was walking cautiously and appeared to be visually impaired. Administration of B₁ hydrochloride continued over the next 4 days and approximately three weeks elapsed before this animal recovered completely. A blood sample was taken from this animal on day 2 of treatment with B₁ (February 6). This animal became anorectic again on March 3, but recovered prior to any treatment. A blood sample taken from this animal at this time (March 3) was analyzed for levels of Cu, zinc (Zn), iron (Fe) and B₁. Another blood sample was taken from the same animal on April 25.

Experimental Study

Since the sick animal responded readily to B₁ administration, diagnosis of PEM was confirmed on February 5, and a collaborative study was initiated to monitor the trace mineral [Cu, Zn, Fe] and the B₁ status of this herd. Blood samples were taken (sampling 1) for measurement of concentrations of Cu, Zn, Fe and B₁ from approximately 70% of the animals in both pens on February 6. Samples of all feed ingredients, drinking water and mineral mix

(Table 2) were taken for analysis of Cu, Zn, Fe, manganese (Mn), molybdenum (Mo), selenium (Se) and total sulfur (S). Based on analysis of trace minerals (Table 1 and 2), total intake of Cu by the animals was found to be inadequate (Table 3). A custom made micromix (Table 4) to supplement the existing mineral supplement was advocated on April 2, 1987. Feeding of this newly mixed supplement commenced on April 5, 1987. Three weeks later, samples of feed, water and blood (sampling 2) were collected (on April 25) as outlined previously, from approximately 34% and 27% of the animals in north and south pen respectively.

Analytical Techniques: Concentrations of Cu, Zn and Fe in plasma were determined after dilution with deionized water (v/v; 1:3) followed by precipitation with 1 volume of trichloro-acetic acid (TCA) (20%) and centrifugation. The plasma supernatants so obtained and farm water samples were measured directly on an atomic absorption spectrophotometer (AAS) (Perkin Elmer, model 5000). Concentrations of Cu, Zn, Fe, Mn, Mo, and Se from samples of forage, barley, canola meal and mineral supplements were also measured on AAS after digestion with a nitric: perchloric acid (3.5:1 v/v) mixture.

Levels of S in forage, barley, canola meal and mineral supplements were analyzed by induction coupled plasma emission spectroscopy (ARL Model 3410) after digestion in nitric and perchloric acids as described by Blanchard et al. (11).

Determinations of blood B₁ was carried out by the method of Olkowski et al (unpub.), a modification of the method of Sarett and Cheldelin (12).

Statistics: Student's t-test (13) was used to determine pre- and post-

supplementation of micropremix (main effect) on concentrations of Cu, Zn, Fe in plasma and B₁ in blood. The secondary effects between breeds (Simmental-crosses and others), and pens (north and south pen) were tested using Analysis of Variance (ANOVA) and Duncan's multiple range test (14). Differences of $p < 0.05$ are reported as statistically significant.

Results

Composition of diets and trace mineral intakes: Table 2 shows that Cu and Zn had been omitted inadvertently by the manufacturer from the mineral mix. Based on feed analysis (Table 1) and a daily consumption of 2.72 kg forage, 4.1 kg barley, 0.91 kg canola meal, 70 g of mineral supplement (Table 2), and a water intake of 18 L (2), we calculated the daily intake of Cu, Zn, Fe and Mo by each steer in the north pen to be 50, 169, 1806 and 0.92 mg respectively. Intake of S via feed and water were 18.6 and 7.9 g respectively. Values for steers in the south pen were approximately 10% less than values for steers in the north pen. Based on 90% feed DM, we calculated the resulting concentrations of Cu, Zn, Fe, Mo to be 7.1, 31, 257 and 0.13 mg/kg of feed DM (Table 3). Similarly intake of S was 0.38% feed DM (Table 3).

Supplementation with a micropremix (initiated on April 5) increased the daily intakes of Cu and Zn by several fold (Table 4). This was most apparent for animals in the north pen because of their higher consumption of this supplement. The intakes of other microminerals also increased but only slightly, whereas the intakes of macrominerals including S did not change to any great extent following supplementation of the micropremix (Table 3).

Growth and general appearance of animals: Examination of the herd on the date of the first blood sampling (Feb. 6) showed evidence of Cu deficiency including lack of desire to feed, unthriftiness, and loss of condition with typical dull, rough hair coat and loss of pigment in hair. (Figure 1).

Three weeks of supplementation with the micropremix was sufficient to improve markedly the overall condition of the animals. All animals showed improved body condition, weight gain, and return of normal coat lustre and color of hair (Figure 2).

Copper status of calves: Copper status of calves at the first sampling as assessed by concentration of plasma Cu, was low (Table 5). Concentration of Cu in plasma of less than 0.3 mg/L has been associated with Cu deficiency in cattle (13). In this context approximately 45% of the animals were Cu deficient (Figure 3). Animals in the north pen appeared to be the most severely affected. Simmental crosses had lower concentration of plasma Cu than the other breeds in the herd. As expected, supplementation of the micropremix resulted in a significant increase ($p < 0.01$) in concentration of Cu in plasma of all steers except the Simmental crosses in the south pen. The latter result may not represent true Cu status of this group since only 2 Simmental crosses from the south pen were sampled at this time. At sampling 2, following supplementation with the micropremix, only 3% of the animals exhibited Cu levels in plasma less than 0.3 mg/L.

Concentration of Cu in plasma of the steer which developed PEM was 0.35 mg/L at sampling 1 but declined to 0.33 on March 3. Three weeks of supplementation with the micropremix increased concentration of Cu in plasma to 0.55 mg/L.

Iron status of calves: The level of consumption of Fe before and after supplementation with the micropremix were similar (Table 3). The concentration of Fe (mean $\pm$ SD) in plasma of cattle after premix supplementation at sampling 2 were significantly lower (2.15 ± 0.42 mg/L) (Mean $\pm$ SD) ($p < 0.05$) compared to the values at sampling 1 (2.73 ± 0.47 mg/L).

Zinc status: Consumption of Zn increased 3 to 4-fold after supplementation with the premix, but the mean plasma Zn concentration of all animals increased only slightly ($p > 0.05$); 1.44 ± 0.26 mg/L at sampling 2 compared with 1.37 ± 0.25 mg/L at sampling 1.

Thiamine status: Mean ($\pm$ SD) blood B₁ concentration at sampling 1 (prior to micropremix supplementation) was 49.2 ± 14.9 ug/L. Approximately 30% of animals in the herd at this time had concentrations of B₁ less than 40 ug/L in blood (Figure 4). Levels in Simmental cattle and other breeds were similar. Concentration of B₁ in blood increased significantly ($p < 0.05$) in all cattle after micropremix supplementation to 128.2 ± 41.7 ug/L. The shift in blood B₁ concentration of the herd following micropremix supplementation is illustrated in Figure 4.

Only the steer which developed PEM received B₁ supplementation. After a single injection of B₁ the concentration of B₁ in blood of this animal increased to 320.3 ug/L, but it declined to 36.1 ug/L four weeks later. No supplementation of B₁ was given after February 10, but three weeks of supplementation with the micropremix alone increased the concentration of B₁ in blood to 96.9 ug/L in this steer.

Discussion

It is evident from the investigation reported here that a combination of excessive intake of S, and a low dietary intake of trace minerals, especially Cu, is detrimental to the B₁ status in cattle. Based on analysis of B₁ in blood of cattle in this study, it might be expected that on the Prairies there are many cattle which suffer from subclinical deficiency of B₁. Supplementation of trace minerals alone, resulted in a significant improvement in both the B₁ and Cu status of cattle exposed to an above-normal exposure of S. Although PEM has been associated with a high concentration of S in the diet (6) and drinking water (8), to our knowledge this is the first time a trace mineral-responsive B₁ deficiency has been documented. We believe that the events leading to a deficiency of B₁ as reported here are due to a series of complex interactions of micro-minerals (Cu, Zn, Fe, Mo), macro-minerals (S) and B₁.

Interactions among Cu, Zn, Fe, Mo, and S are widely recognized (15). The bioavailable fraction of each of these elements can be regarded to be "in balance" with that of the other four, and hence the changes in concentration of one would distort the metabolism of the others (15). In this context, it is notable that concentration of Zn in plasma was lower at sampling 2 in spite of a three-to-four-fold increase in intake of dietary Zn after supplementation with the micropremix. This may have resulted from a decreased absorption of dietary Zn because the concentration of Cu in the diet of animals in the two pens increased from 7 to 37.0 (south pen) and 57.5 (north pen) mg/kg during this period (Table 3). Similarly, the concentration of Fe in plasma of animals was also significantly lower at sampling 2. This is not surprising since excess dietary Cu, relative to Fe, is known to reduce

the Fe status in cattle, and vice versa (16). It has been shown that 250 mg/kg DM of Fe in the diet can induce Cu deficiency in cattle (17) and an enhanced accumulation of Fe has been noted in such animals (18). Copper deficiency produces anemia and a characteristic response to anemia is an enhanced efficiency in Fe absorption (19). Thus a relatively higher concentration of Fe in plasma observed at sampling 1 (prior to supplementation with the micropremix) was probably due to an enhanced absorption of Fe by Cu-deficient animals from the diet which contained low Cu, moderately high Fe, and marginal Zn.

In ruminants, both inorganic and organic S compounds are reduced to sulfides by rumen bacteria. Sulfides bind with a variety of divalent cations to form cationic sulfides. Affinity of S for Cu is higher than other cations and the solubility product of Cu sulfide is extremely low (3.48×10^{-38}). In addition, thiomolybdates (TM) which form in the rumen at moderate to high intakes of S and Mo, form unavailable Cu-TM complexes which exert effects both in the gut and systemically (7), further reducing the availability of Cu to the animal. Breed differences in Cu metabolism are recognized and Simmental cattle appear to be more susceptible to Cu deficiency than other breeds (7). This is supported by the present study in that the concentration of Cu in plasma of the Simmental crosses were lower than those in the other breeds (Table 5). Hypocupremia was evident at sampling 1 (Table 5) with approximately 45% of the animals deficient in Cu (Figure 3). Depigmentation and rough hair coat observed at this time (Figure 1) are typical manifestations of Cu deficiency (20). Reversal of these signs within 3 weeks of supplementation with the micropremix suggest synthesis of Cu-dependent enzymes such as polyphenyloxidases which are involved in pigmentation and

keratinization of hair. It was not surprising to find an improvement in growth and body condition after supplementation with micropremix. Significant increases in weight gains, have been reported in previously deficient cattle when supplemented with Cu (21) and B₁ (22). Therefore it is reasonable to believe that the improved animal performance observed in the present study was due to a combined improvement in Cu and B₁ status in these animals.

Recent evidence that excess dietary S also exerts an effect on availability of B₁ in ruminants (10) suggests the possible occurrence of a complex interaction of B₁-S-divalent cations in the rumen. If this is true, a low concentration of divalent cations in the diet would indirectly result in a relatively higher concentration of 'free' sulfide in the rumen and this may create a B₁ deficiency. We believe that this may have been the reason for the depleted levels of blood B₁ in blood (sampling 1) observed in the present study prior to supplementation with the micropremix. Conversely, if the diet is supplemented with normal or a slight excess of cations, especially with Cu, it would result in formation of cationic sulfides. This would lower the concentration of free sulfide in the rumen (23), and thereby alleviate the effects of S-induced B₁ deficiency. This is probably the most likely explanation for the significant increase in levels of B₁ observed in blood of animals in the present study after supplementation with the micropremix. The response was immediate, since an improvement in B₁ status was observed within 3 weeks of supplementation. This improvement in B₁ status occurred in spite of no change in basal diet, drinking water supply or any supplementation with B₁.

Clinical signs of B₁ deficiency have been divided broadly into those attributable to general metabolic disorders, which may be observed at an early

stage, and those related to disorders of the central nervous system (24). It is interesting to note that the clinical signs listed as metabolic disorders of B₁ deficiency such as transient scouring, reduced growth rate, anorexia, general unthriftiness and reproductive disorders are also common signs of chronic Cu deficiency. We believe that under field conditions deficiencies of both Cu and B₁ may exist concurrently in cattle having access to excessive levels of S. Instances in which high S-related PEM is diagnosed, conventional B₁ therapy (25) is beneficial and clinical recovery occurs within one to three days. However, as observed in the present study, the elevation of concentration of B₁ in blood after IV B₁ therapy is only temporary. As long term therapy, we recommend that herds exposed to high intakes of S also be supplemented with Cu up to 50 mg/kg feed DM. This could be expected to alleviate deficiencies of both Cu and B₁.

In a previous experiment, two out of eight cattle fed 0.5% S and 10 mg Cu/kg DM in the diet, developed PEM within 6 weeks. Concentration of B₁ in blood of affected animals were 14.7 and 23.5 ug/L (Gooneratne et al, unpub. obs.). Based on our experience, we believe that a concentration of less than 40 ug/L of B₁ in blood could be considered as marginal. In this context approximately 30% of the animals in the present study were deficient at sampling 1. Depression of concentration of B₁ in blood has been reported previously in cattle fed diets adequate in Cu but supplemented with 0.72% sulfate (10). Since ruminants do not require B₁ in their diet, one or several of following mechanisms may have been responsible for the lower levels of B₁ in plasma as observed in the present study: inadequate microbial synthesis of B₁, impaired absorption and utilization of B₁, presence of B₁ anti-metabolites, lack of apoenzyme, increased metabolic demand for B₁ or increased

rate of B₁ excretion (3). It is not clear which of these pathways are involved in the induction of S-related B₁ deficiency. However, there is some evidence that diets high in S decrease the amount of B₁ entering the duodenum, probably due to a reduced synthesis of B₁ (10), and increase excretion of B₁ (Olkowski et al, unpub. obs.). High intakes of S decrease ruminal, duodenal and fecal pH (26). Ruminal acidosis appears to establish conditions conducive to development of PEM (27), such as enhanced production of the B₁-destroying enzyme, thiaminase (1). It is reported that B₁ and other related compounds can repress extracellular enzyme, thiaminase (28), and hence a critical concentration of extracellular B₁ in rumen fluid may be an important factor in the control of thiaminase production. We believe that future research needs to be directed at understanding: (i) whether excess S induces subclinical lactic acidosis in rumen, (ii) whether thiaminase-producing organisms are selected or encouraged preferentially by high intakes of S, and (iii) how high intakes of S increase urinary B₁ excretion. Experiments are in progress to test these hypotheses.

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TABLE 1

Trace mineral and sulfur concentrations^a in feed and deep well water

Sample	Cu	Zn	Fe	Mn	Mo	Se	S ^b
Forage	13.8	22.0	412.7	43.3	0.12	0.60	0.23
Barley	3.9	21.3	51.4	15.5	0.12	0.64	0.13
Canola meal	7.5	52.2	174.0	51.9	0.08	1.71	0.73
Deep well water (sampling 1) (February-6, 1987)	0.01	0.04	0.02	0.007	0.18	0.006	437
Deep well water (sampling 2) (April 25, 1987)	0.004	0.04	0.02	0.005	0.05	0.006	442

^a Cu, Zn, Fe, Mn, Mo and Se concentrations in feed and water are expressed as mg/kg DM and mg/L respectively.

^b S concentration in feed and water are expressed as % DM and mg/L respectively.

TABLE 2

A comparison of manufacturer's guaranteed analysis and laboratory analysis of cattle mineral mix fed to cattle prior to micropremix supplementation

Units	Mineral	Guaranteed analysis by manufacturer ^a	Laboratory analysis of mineral mix ^b
%	Ca	14.0	11.4
	P	14.0	11.6
	Na	8.0	11.6
	S	c	0.57
mg/kg	Cu	3,000	32
	Zn	5,025	332
	Fe	1,000	4941
	Mn	c	155
	Mo	c	0.5
	Se	c	0.32

^a Guaranteed analysis printed on label by manufacturer. This feed was also guaranteed to contain 1600 mg/kg of iodine, 100 mg/kg of cobalt, and a minimum of 100,000 IU/kg of vitamin A, 50,000 IU/kg of vitamin D₃ and 100 IU/kg of vitamin E.

^b Analysis was carried out by the Feed Testing Laboratory, University of Saskatchewan, Saskatoon.

^c not available.

TABLE 3

Copper, Zn, Fe, Mo and S intake by cattle prior to and after micropremix supplementation

	Pen	Mineral intake				
		Cu	Zn	Fe	Mo	S
		(mg/kg DM)				
Prior to Supplementation ^a	North	7.1	31	257	0.13	0.38
	South					
After Supplementation ^b	North	57.5	126	268	0.18	0.38
	South	37.0	86	245	0.16	0.38

^a These were based on average daily intakes of 2.72 kg forage, 4.1 kg barley and 0.9 kg canola meal (see Table 1 for analysis) and 70 g mineral mix 1 (prior to supplementation) (see Table 2), by steers in the north pen, and 10% less by steers in the south pen. But since the concentrations of minerals are expressed in terms of feed DM intake, the values are similar for both pens. Daily water intake was assumed to be 18 L per animal.

^b Feed intake was similar to above^a but intake of custom made micropremix (see Table 4) varied between the animals in the two pens; 133 g by steers in the north pen compared to 78.5 g by those in the south pen.

TABLE 4

Manufacturer's guaranteed and laboratory analysis of custom-made micropremix advocated to supplement the existing mineral mix after diagnosis of trace mineral deficiency.

Mineral	Guaranteed analysis	Laboratory	Daily intake of mineral/animal ^b (mg)	
	<u>by manufacturer^a</u> (mg/kg)	<u>analysis</u> (mg/kg)	<u>after micropremix supplementation</u> North Pen	South Pen
Cu	50,000	69,658	359	212
Zn	140,000	131,878	700	413
Fe ^c	d	6,967	429	254
Mo ^c	d	65	0.36	0.21
Mn ^c	d	519	15.0	12.0
Se	400	480	2.48	1.60
S ^c	d	3.18%	0.61g	0.36g

^a Minimal guaranteed analysis by supplier. This micro-premix also contained 3×10^7 IU/kg of vitamin A, 4×10^6 IU/kg of vitamin D₃ and 10,000 IU/kg of Vitamin E.

^b Mineral supplementation to cattle from April 5 consisted of a mixture of 1.6 kg of the micropremix, 25 kg of mineral mix (see Table 2), 8.2 kg of calcium carbonate, and 6.8 kg of blue salt (sodium chloride, iodine, cobalt). Daily intakes of minerals have been estimated from intakes of 133 g/d and 78.5 g/d of this mixture by steers in north and south pen respectively.

^c These minerals were not recommended for addition to this micromix but appeared as contaminants.

^d not available

TABLE 5

Plasma Cu concentration^a of calves prior to and after micropremix supplementation.

Plasma Cu concentration (mg/L)					
	<u>Both Pens</u>	<u>North Pen</u>		<u>South Pen</u>	
		Simmental	Others	Simmental	Others
		<u>CROSSES</u>		<u>CROSSES</u>	
Prior to	0.42 ^d	0.34 ^f	0.38 ^f	0.42 ^{fg}	0.48 ^g
Supplementation ^b	±0.16	±0.11	±0.14	±0.16	±0.16
	(n-77)	(n-17)	(n-21)	(n-7)	(n-32)
After	0.68 ^e	0.72 ^h	0.70 ^h	0.45 ^{fg}	0.67 ^h
Supplementation ^c	±0.15	±0.14	±0.19	±0.21	±0.10
	(n-32)	(n-8)	(n-10)	(n-2)	(n-12)

^a Results are expressed as mean ± SD^b Prior to supplementation refers to sampling 1 on February 6, 1987.^c After supplementation refers to sampling 2 on April 25, 1985, taken 3 weeks after commencement of micropremix supplementation.^{d,e} Values with different letter superscripts differ significantly (p<0.01) (Students test)^{f,g,h} Values with different letter superscripts differ significantly (p<0.05) (Duncan's Multiple Range Test)

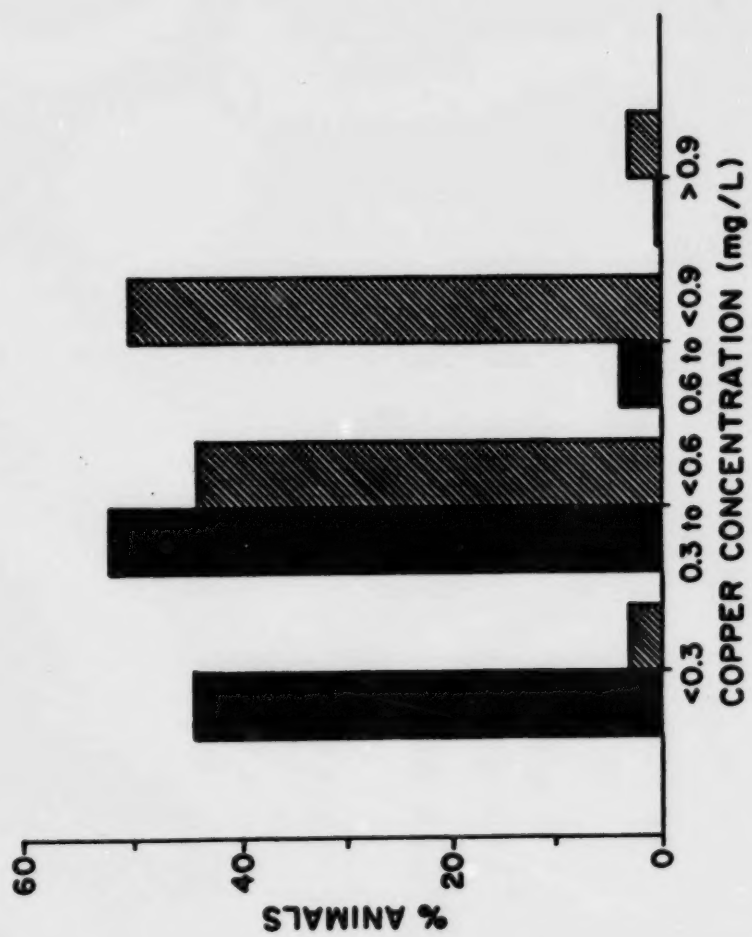
LEGEND FOR FIGURES

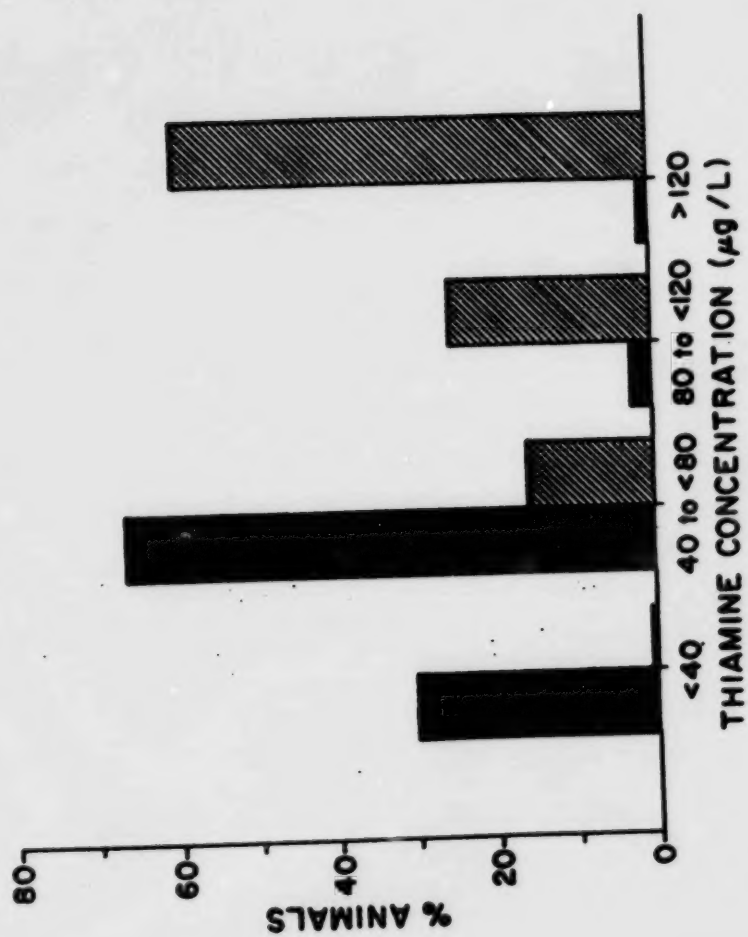
Figure 1: Shows the appearance of steer (No.28) (which developed polioencephalomalacia) prior to trace mineral supplementation. Note the depressed state of the animal and its dull, rough and depigmented coat.

Figure 2: Appearance of the same animal (steer 28) after three weeks of trace mineral supplementation. Note the marked improvement in body condition, coat texture and coat color.

Figure 3: A comparison of plasma copper concentration in cattle prior to (■ , sampling 1), and after (▨ , sampling 2) micropremix supplementation.

Figure 4: A comparison of blood thiamine concentration in cattle prior to (■ , sampling 1), and after (▨ , sampling 2) micropremix supplementation.





Part 1. Sulfur Induced Polioencephalomalacia in Sheep: Some Biochemical Changes

S.R. Gooneratne, A.A. Olkowski and D.A. Christensen

Abstract

The effect of high dietary sulfur (S) supplementation on blood thiamine (B_1) concentration, biochemical indices of liver, muscle and kidney damage and selected plasma electrolytes were studied in 6 sheep. Three of these sheep received additional 230 mg thiamine/kg diet (Gp. 2). After approximately 2.5-3 weeks on this diet, all 3 sheep in the non- B_1 -supplemented group (Gp. 1) showed loss of appetite and developed milk neurological signs; depression, intermittent signs of excitation and head pressing. Increases in blood B_1 concentrate and plasma creatine kinase (CK) and aspartate amino-transferase (AST) were observed during this time in all affected animals. Clinical signs lasted only for 2-5 d and both animals recovered completely. Sheep in Gp. 2, by comparison, were clinically normal throughout the experiment, but all of these animals also had elevated blood B_1 concentration and plasma CK activity was observed at the 3 wk sampling.

Compared with mean baseline values the following plasma electrolyte changes occurred. Plasma magnesium concentration of Gp. 1 sheep were elevated during 2.5-3 wk and 6 wk sampling but declined significantly ($P < 0.05$) to low normal levels thereafter. Magnesium concentrations of Gp. 2 sheep were low at the beginning but progressively increased during the course of the experiment. At necropsy, brain lesions suggestive of polioencephalomalacia (PEM) were observed in all sheep but were most marked in Gp. 1 sheep which did not receive supplemental B_1 .

The present study has shown that polioencephalomalacia can be experimentally produced in sheep given high levels of S in the diet.

Biochemical parameters and plasma electrolytes monitored in this study are in agreement with those values reported in both naturally occurring and amprolium induced PEM in ruminants and horses. Since brain lesions indicative of PEM were observed in both B₁ supplemented and unsupplemented groups it is speculated that this syndrome may be caused by a direct toxic effect of S, S metabolites or B₁ antimetabolites in the brain rather than by an invivo B₁ deficiency per se.

Impact and Significance for Saskatchewan Agriculture

Permanent brain and neurological system damage may occur when ruminants are exposed to a thiamine deficiency. It is unlikely that full recovery of brain function will result from supplementation with thiamine. It is therefore necessary to avoid circumstances in which a severe thiamine deficiency occurs or where the deficiency occurs for more than a brief period.

INTRODUCTION

Polioencephalomalacia (PEM) is a neurologic disease of ruminants. It is commonly associated with feeding of a high level of concentrate and/or a low amount of roughage (1). Recent studies have shown that diets (2) or drinking water (3,4) containing high levels of sulfates also predispose cattle to PEM, but little is known of the mechanisms or the biochemical changes associated with S induced PEM in ruminants. Although biochemical findings on experimental and field cases of PEM are consistent with lowering of thiamine (B₁) status, there is no complete agreement in the literature that the blood B₁ levels are always reduced in affected animals. Studies on PEM have shown that blood B₁ levels may be decreased (5) while other reports have shown it to be within normal limits (6,7) or even elevated (8). The reason for such discrepancy is not clear but if PEM is induced by analogues such as amprolium or by B₁ antimetabolites blood B₁ concentrations may not always be low.

Magnesium (Mg) is a co-factor of several B₁ containing enzymes (9). Metabolic interactions between minerals, and between minerals and other components in the diet and body fluids are well recognized (10). Changes in plasma concentration of electrolytes, sodium (Na), potassium (K), calcium (Ca), phosphorus (P), magnesium (Mg), chloride (Cl) have been reported in naturally occurring PEM (11) and in amprolium induced B₁ deficiency in sheep (6) and horses (12). Such information is lacking in S induced PEM in ruminants. Excess S in the diet has been shown to induce muscular dystrophy in ruminants by increasing excretion of selenium (Se) in urine (13). Recent studies have shown an association between B₁ deficiency and hepatorenal syndrome in humans (14). The objective of the present study was to

experimentally induce PEM in sheep by feeding high S and to examine changes in blood B₁ concentration, certain plasma electrolyte concentrations and biochemical indices of liver, muscle and kidney damage during the progression of the disease.

MATERIALS AND METHODS

ANIMALS, DIETS AND BIOCHEMICAL ANALYSES

Six, 8-week old lambs of nondescript breed of mean body weight 12 - 15 kg were used. All lambs were fed a barley-soybean meal-alfalfa based diet containing either high (H) S (0.63%) - basal (L) B₁ (13.7 mg/kg DM) (Group 1, n=3) or HS-HB₁ (230 mg/kg DM of B₁) (Group 2, n=3). Measurement of blood B₁ (15) and plasma concentrations of creatine kinase (CK) (16), aspartate aminotransferase (AST) (17), γ -glutamyl transferase (GGT) (18), creatinine (CRT) (19), urea (20), sodium (Na), potassium (K), chloride (Cl), calcium (Ca), magnesium (Mg), were carried out on using a Discrete Analyzer with Continuous Optical Scanning (DACOS, Coulter Electronics Inc. Hialeah, Florida) prior to initiation of the trial, at every 3 wk period thereafter, and on days when an animal showed neurological symptoms until the termination of the experiment. Since these animals were a part of a bile collection experiment, after 8 wks on the diets, ligation of bile duct, and cannulation of gall bladder and mid-third of duodenum were performed on all sheep as described by Caple and Heath (21) except sheep #81 in Gp. 2. Clinical appearance and vital neurological signs were monitored daily.

STATISTICS

Statistical analysis was carried out using General Linear Model analysis

of Variance (GLM ANOVA) from microcomputer package [Number Cruncher Statistical System (NCSS)]. Fisher LSD (22) test was used to determine statistical significance of means between the treatment groups.

RESULTS

CLINICAL SIGNS

All animals in Gp. 2 which received supplemental B₁ appeared clinically normal throughout the experiment. In contrast, all three sheep fed the HS-LB₁ diet (Gp. 1) showed loss of appetite and mild neurological signs such as mild depression, intermittent signs of excitement and head pressing within 2-3 wks of commencement of the experiment. Both sheep recovered completely within 2-5 d, but sheep #47 died suddenly on day 37 of the experiment. No signs of illness were observed in the other 2 animals in the group during the remainder of the experiment. Necropsy and/or histological findings indicative of PEM, such as cortical and mid-brain focal necrosis (23) were seen in all sheep. But lesions were most severe in sheep #47 which died and in the remaining Gp. 1 sheep (Olkowski et al, unpublished results).

BLOOD THIAMINE AND PLASMA ENZYME CHANGES

Blood B₁ concentration of all lambs were within the reference limits (5) prior to the start of the experiment (Table 1) but 2 sheep (#47 and #72) in Gp. 1 which exhibited neurological signs had increased levels of up to 242 and 173 mg/L at the height of signs (2-3 wks). Aspartate aminotransferase activity was increased in all lambs in Gp. 1 at this time but CK activity was increased only in 2 lambs. These changes were most marked in sheep #47 (Fig. 1). Samples taken at 3 wk from apparently clinically normal sheep in Gp. 2

also showed markedly elevated blood B₁ concentration (Table 1). Plasma CK was elevated in 2 of 3 lambs in this group. These changes were most marked in lamb #78 (Fig. 1). Plasma AST activity was also markedly elevated in this lamb during this time. Plasma GGT activity remained within normal limits in all 6 animals during the initial 6 wk period.

Blood B₁ concentration of lambs in both groups returned to within normal limits after 3 wk. But plasma CK was elevated even at 6 wk. Both CK and AST activities fluctuated during the remainder of the experiment. From 9 wk onwards GGT activities were increased in all sheep except lamb #81 (Gp. 2) which was not cannulated. Plasma creatinine and urea levels of all animals remained within the normal limits of 69-105 $\mu\text{mol/L}$ and 0-10 mmol/L respectively.

Blood B₁ concentration of a sample from sheep #47 in Gp. 1, taken 24-48 h prior to its death was within the normal range. But plasma CK activity was elevated (588 U/L). Both AST and GGT activities of this animal were within normal limits at this time.

PLASMA ELECTROLYTES

Changes in plasma concentration of Na, K, Cl, Mg, Ca and P of sheep in both groups during the course of the study are given in Table 2. Potassium concentrations remained within reference limits (4.6 - 7.0 mmol/L) in all lambs in Gp. 1 but all lambs in Gp. 2 exhibited hyperkalemia (up to 10 mmol/L) during wk 3. This persisted in 2 of 3 animals until wk 6 but decreased thereafter. Changes in Na were minor but Cl concentrations declined significantly to below normal level at wk 9 in all Gp. 2 sheep.

Among Gp. 1 sheep, plasma Mg concentrations increased to above normal

levels during 2-3 and 6 wk sampling but declined significantly ($P < 0.05$) to low marginal levels thereafter. On the other hand Mg concentration of sheep in Gp. 2 was low at the beginning of the experiment (normal range - 0.90 - 1.26 mmol/L) but increased during the course of the experiment. Slightly above normal levels were detected at wk 9 sampling.

Plasma Ca concentration remained within normal limits in all sheep throughout the course of the experiment. Plasma P concentrations were above normal (normal range 0.82 - 2.66) in all animals at the onset of the experiment. Increases in plasma P were observed in both groups during the initial 6 wk. The levels of P in Gp. 1 sheep declined thereafter but the plasma P concentration of lambs in Gp. 2 continued to increase throughout the experiment and high levels were observed at the termination of the study.

DISCUSSION

The present study has shown that PEM can be experimentally produced in sheep given high levels of S in the diet. This confirms observations recorded from field investigations that PEM can be caused by consumption of either high S (as sulfate) in the ration (2) or in the water (3,4,24). This is also consistent with finding that invitro sulphite is capable of cleaving B₁ in aqueous solutions (25). However Edwin et al (26) were unable to produce PEM in young sheep by feeding 15 g of sodium sulfite up to one year. In the present study excess S was fed only as sulfates and it is not known whether the ionic configuration of this form of S had an influence on the development of PEM. In the present study, neurological signs indicative of B₁ deficiency were not observed in B₁ supplemented sheep. However, brain lesions suggestive

of PEM (Olkowski et al, unpub. results), were seen in all 3 sheep supplemented with B₁ but the lesions were far less severe (23). This suggests that factors other than deficiency of B₁ may be responsible for the development of brain pathology of sheep fed high S diets.

Increase in blood B₁ concentration during the appearance of early neurologic symptoms of PEM have not been reported previously. However above normal blood B₁ levels have been reported in clinically normal animals within 1-2 wks of feeding amprolium, a B₁ analogue to calves (8). This author used the thiochrome method for estimation of blood B₁ concentration and the possibility that amprolium in blood may have interfered with the assay (7) cannot be ruled out. Slight elevations of blood B₁ accompanied by corresponding increases in urine B₁ have also been reported in sheep within 2-3 weeks of feeding a bracken rhizome diet (27). In the latter study a second elevation of both blood and urine B₁ levels was observed at 4-5 wks of feeding bracken. Nervous signs were not detected on either occasion. Increased excretion of B₁ in urine has been reported in calves given a high carbohydrate diet (28), or amprolium (8). We have observed (Olkowski et al, unpub obs) similar increases in urine B₁ in all sheep fed high S. As expected such increases were most marked in sheep supplemented with B₁.

The relevance of the increase in blood B₁ concentrations during early stages of the disease and its relationship to the appearance of mild neurological signs as observed in the present study is not clear. This is because extremely high blood B₁ concentrations were also observed in apparently normal B₁ supplemented sheep during the same time. But sheep fed low S diets do not show such elevations in blood B₁ concentration (Olkowski et al, unpublished results). Origin of elevated B₁ in blood of sheep fed high S

is not known but it is possible that leakage may have occurred from either the muscle and/or the liver since both plasma CK and AST activities were elevated. Hepatic lesions have been inferred from clinical pathology data of amprolium induced PEM in horses (12), but it is unlikely that such marked liver damage occurred in sheep in the present study since normal GGT levels occurred during periods of CK and AST elevations. Elevated levels of both AST and GGT were observed in subsequent weeks; wk 9 and 12 in Gp. 1 sheep and wk 9 in Gp. 2 sheep. This probably occurred from a mild cholestasis as a result of the indwelling gall bladder catheter in these animals since such an elevation was not observed in the uncannulated sheep #81 in Gp. 2.

Although muscle does not store B_1 to any great extent a marked elevation in plasma B_1 due to leakage from muscle is still a possibility because of the large muscle mass in the body. Increases in plasma CK have been previously reported in amprolium induced PEM in calves (8) but only during periods of violent clonic spasms. Blood B_1 concentration of such animals were only slightly elevated. But the possibility that blood B_1 concentrations may have peaked in these animals prior to measurement of CK cannot be discounted. Evidence of cardiac lesions, and associated functional cardiac impairment have been reported in B_1 deficiency in the cat (29) and in ruminants (30). The technique used to determine CK in the present study did not differentiate whether its origin was solely from skeletal or cardiac muscle or a combination of both. Creatine kinase is a cytosolic enzyme in muscle. High plasma CK values are indicative of either an increase in muscle cell permeability or acute damage to muscle. Increases in plasma CK have been reported in a variety of muscular disorders including Se deficiency associated nutritional muscular dystrophy (NMD) in calves (31) and NMD in lambs (32,33). It is not

known whether feeding of a high S diet to sheep precipitates an early Se deficiency but muscular dystrophy has been reported in sheep fed high S diets (13).

Increased plasma CK as observed in the present study does not exclude the possibility of nervous damage being the cause of CK release since elevations of CK have also been reported to occur in a variety of diseases of the nervous system in man (34) and animals (35). Typical histological lesions of PEM were observed in the brains of all sheep but the lesions were most severe in the animals not supplemented with B₁ (23). It has been postulated that in encephalomalacia, plasma CK of brain origin is not elevated since the blood brain barrier (BBB) is not altered. But starvation and stress a common occurrence during height of nervous signs significantly disrupts BBB (36). Dubo et al (34) and Smith and Healy (35) attributed increased CK observed in nervous disorders to abnormal muscle contractions which are a part of the neurological syndrome. Although this may have occurred in Gp. 1 lambs which exhibited mild neurological symptoms, it is unlikely to be the explanation for the rise in CK observed in Gp 2 lambs since neither neurological signs nor an increase in muscular activity were observed in these sheep during the time of CK rise.

Creatine kinase is eliminated rapidly from blood (37) and the half-life of CK derived from sheep in only 62 mins (32). Therefore the presence of high CK levels observed in the present study at 3 and 6 wk suggest insult to muscle during these times although more frequent sampling would have confirmed whether such damage was persistent or not. However blood B₁ concentration of all lambs at 6 wks were within normal levels. If blood B₁ originated from muscle, the return of blood B₁ to within normal levels in spite of continued

high CK levels is not known. It should be noted that most of B_1 is associated with cellular mitochondria and only the amount of B_1 present in cytosol would leak out during permeability change or muscle damage. It can be speculated that the initial increase in muscle permeability may have resulted in leakage of almost all of the muscle cytosolic B_1 into the bloodstream leaving little or no B_1 for leakage during subsequent changes in muscle permeability. The marked increase in blood B_1 concentration observed in B_1 supplemented sheep (Gp. 2) probably indicates leakage from relatively higher stores of B_1 in muscle and/or liver of these sheep compared to the levels in sheep in Gp. 1. Since both CK and blood B_1 returned to baseline values after 6 wk it is assumed that the sheep may have recovered from an initial insult caused by high S, or adapted to the high S diet by this time.

Changes in plasma electrolyte concentration observed in the present study are in agreement with previous reports on ampolium induced PEM in cattle (8, 11). No marked changes were observed in either plasma Na or Ca concentration. The decline in plasma Mg in Gp. 1 sheep as opposed to an increase in Gp. 2 sheep during terminal stages of the experiment (wk 12) is consistent with finding that the metabolism of B_1 and Mg are interdependent (9). Magnesium is a co-factor of B_1 dependent enzymes, pyruvate dehydrogenase, -ketoglutarate dehydrogenase and transketolase. The reason for the marked increase of plasma Mg concentration at 2.5-3 and 6 wk sampling in Gp. 1 sheep is not clear. Blood B_1 and plasma CK were also elevated in these animals during this time. It has been shown that animals displaying increased muscular activity may have above normal Mg concentration in blood due to a release of Mg from tissues (38). Similar increases in plasma Mg have been recorded in feedlot steers fed an all-concentrate barley based diet (39) and during wks 1, 2 and wk 5

following amprolium treatment in cattle (6). The CK was not measured in the latter study. It is unlikely that Mg leaked out from the muscle since Gp. 2 animals which showed elevated levels of CK and markedly elevated blood B₁ concentration at wk 3 sampling showed only a marginal increase in plasma Mg.

It is notable that hyperkalemia and elevated blood B₁ occurred concurrently in Gp. 2 sheep during wk 3 and this was also observed at wk 6. Highest concentration of plasma K was observed in Gp. 1 lambs at 6 wk when plasma CK was still marginally high. Increased serum K concentration has been reported in amprolium treated geldings during the height of clinical signs (12). Sheep in Gp. 1 had low-normal plasma K concentration during terminal stages of the present study. Low plasma K has been reported in calves during terminal stages of PEM (40) but not in adult cattle (41). Little is known about P metabolism in animals affected with PEM. The increases in plasma P levels observed in all sheep during the initial 6 wk is probably related to rapid growth and bone turnover in young animals (42). The reason for decline in plasma P in Gp. 1 sheep and an increase in Gp. 2 sheep which received B₁ supplementation is not known. Decreases in serum P levels have been previously reported in 2 horses receiving amprolium but these animals in addition also received Mg supplementation (12).

In conclusion, changes in several biochemical parameters observed in high S related PEM in sheep appears to be similar to those observed in naturally occurring (11) and amprolium induced PEM in ruminants (6,8,27) and horses (12). It is still debatable whether a localized B₁ deficient state in the brain as suggested by Loew et al (7) is in fact responsible for the appearance of brain lesions and signs of PEM. The present study does not support either a systemic or a localized B₁ deficiency as the cause of high S related PEM in

sheep since both blood B₁ (Table 1) and brain B₁ concentrations (2.2-3.0 ug/g brain wet weight; Olkowski et al, unpub. results) of all sheep at the conclusion of the experiment were within normal range [blood: 18-60 ug/L (5); brain: 1.29-1.82 ug/g wet weight (43)]. Since brain lesions suggestive of PEM were observed in both B₁ supplemented and unsupplemented groups of sheep, it seems likely that a direct toxic effect of either S, S metabolites, or B₁ antimetabolites on the brain could have been responsible for this occurrence. In view of the abundance of high sulfate water available to livestock in Saskatchewan the concept of S induced nervous disorder resembling a B₁ deficiency in ruminants has wide application of economic importance. Further investigation is needed to establish the mechanisms responsible for the occurrence of this syndrome. Early identification of a subpopulation of animals at risk of development of brain damage in high S fed animals would be extremely valuable from the standpoint of clinical management of the problem.

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LEGEND FOR FIGURES

Figure 1 - Changes in blood thiamine concentrations and activities of creatine kinase, aspartate aminotransferase and γ -glutamyl transferase in plasma of a sheep given high sulfur-low thiamine diet (sheep #47) and one given high sulfur-high thiamine diet (sheep #78). The asterisk (*) indicates the time when neurological signs were observed in sheep #47.

TABLE I - Changes in blood thiamine (B₁) concentration, and plasma activities of creatine kinase (CK), aspartate amino transferase (AST) and -glutamyl transferase (GGT) in sheep fed high sulfur with (Group 2) or without (Group 1) supplemental B₁ in the diet.

	<u>Group 1^a</u>					<u>Group 2^b</u>				
	<u>High S - Basal B₁ Diet</u>					<u>High S - High B₁ Diet</u>				
	<u>Time of Sampling (wk)</u>					<u>Time of Sampling (wk)</u>				
	<u>0</u>	<u>2.5-3</u>	<u>6</u>	<u>9</u>	<u>12</u>	<u>0</u>	<u>3</u>	<u>6</u>	<u>9</u>	<u>12</u>
Blood B ₁ concentration (ug/L)	51 ^d ±31	207 ^c ±28	73 ^d ±4	50 ^d ±1	44 ^d ±11	57 ^d ±4	742 ^c ±284	97 ^d ±4	89 ^d ±3	90 ^d ±2
Plasma CK ^e activity (U/L)	312 ^{cd} ±109	513 ^c ±145	386 ^{cd} ±104	276 ^{cd} ±162	118 ^d ±12	245 ±14	750 ±482	654 ±333	93 ±39	280 ±137
Plasma AST ^e activity (U/L)	91 ^d ±1	112 ^d ±11	91 ^d ±4	300 ^c ±166	149 ^{cd} ±11	106 ±21	324 ±240	144 ±9	73 ±22	287 ±126
Plasma GGT ^e activity (U/L)	73 ^d ±5	63 ^d ±6	61 ^d ±4	449 ^c ±4	470 ^c ±44	68 ^d ±2	59 ^d ±4	60 ^d ±5	159 ^d ±8	385 ^c ±43

^a Number of animals sampled from each group was 3 except during wk 6, 9 and 12 in Gp. 1 when only 2 animals were sampled. Sheep #47 from this group died on day 37 of the experiment.

^b Results are expressed as mean ± SD.

^{cd} Means within a row and within treatment followed by different letter superscripts are significantly different (P<0.05).

^e Reference values for enzymes CK, AST and GGT set by the Clinical Pathology Laboratory, Western College of Veterinary Medicine, University of Saskatchewan are <350, 48 to 128 and <70 U/L respectively.

TABLE II - Plasma electrolyte concentration^a of lambs fed high sulfur (S)-normal thiamine (B₁) (Group 1) and high S-high B₁ diets (Group 2).

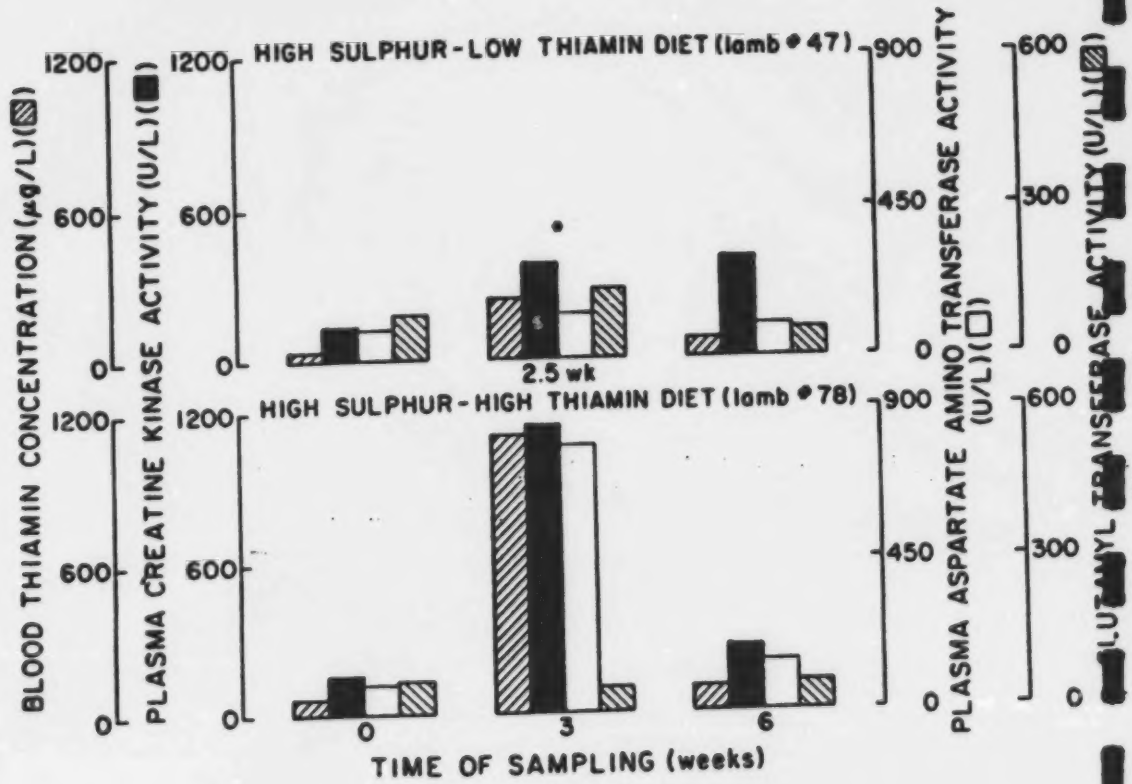
Electrolyte (mmol/L)	Group 1					Group 2				
	High S - Basal B ₁ Diet					High S - High B ₁ Diet				
	Time of Sampling (wk)					Time of Sampling (wk)				
	0	2.5-3	6	9	12	0	3	6	9	12
Sodium ^d	149	148	147	148	147	149	146	143	143	149
Potassium ^d	± 2 5.6 ^{bc} ± 0.6	± 2 5.6 ^b ± 0.1	± 1 6.1 ^b ± 0.3	± 3 4.7 ^c ± 0.1	± 1 4.6 ^c ± 0.3	± 3 5.7 ^{bc} ± 0.4	± 2 7.7 ^b ± 1.3	± 3 8.1 ^b ± 1.2	± 4 4.7 ^c ± 0.7	± 1 5.1 ^{bc} ± 0.5
Chloride ^d	107 ± 2	107 ± 2	107 ± 1	105 ± 1	109 ± 1	106 ^b ± 1	107 ^b ± 1	105 ^b ± 2	89 ^c ± 11	104 ^b ± 1
Magnesium ^d	1.0 ^{bc} ± 0.1	1.3 ^b ± 0.1	1.3 ^b ± 0.2	0.9 ^c ± 0.1	0.9 ^c ± 0.1	0.8 ^c ± 0.1	1.0 ^{bc} ± 0.1	1.1 ^{bc} ± 0.1	1.3 ^b ± 0.3	1.1 ^{bc} ± 0.1
Calcium ^d	2.8 ± 0.1	2.6 ± 0.1	2.6 ± 0.2	2.7 ± 0.1	2.7 ± 0.1	2.8 ± 0.1	2.6 ± 0.1	2.5 ± 0.1	2.4 ± 0.1	2.6 ± 0.1
Phosphorus ^d	2.8 ^c ± 0.4	3.2 ^{bc} ± 0.2	3.6 ^b ± 0.3	2.8 ^c ± 0.1	2.6 ^c ± 0.2	2.9 ^c ± 0.3	3.1 ^{bc} ± 0.3	3.1 ^{bc} ± 0.4	3.7 ^b ± 0.6	3.7 ^b ± 0.3

^a Results are expressed as mean $\pm$ SD. Number of animals sampled from each group was 3 except during wk 6, 9 and 12 in Gp. 1 when only 2 animals were sampled. Sheep #47 from this group died on day 37 of the experiment.

^{bc} Values with different letter superscripts within a row and within treatment differ significantly ($P < 0.05$).

^d Reference values for electrolytes sodium, potassium, chloride, magnesium, calcium and phosphorus as recognized by the clinical pathology laboratory, Western College of Veterinary Medicine, University of Saskatchewan are 143 to 151, 4.6 to 7.0, 102 to 116, 0.9 to 1.26, 2.3 to 2.86 and 0.82 to 2.66 respectively.

FIGURE 1



Part 2. Effects of High Dietary Sulfur on Brain Functions Using Evoked Potentials Technique

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Abstract

Brain stem auditory-evoked response (BAER) is a noninvasive technique used for detecting neurophysiologic abnormalities of the brain stem along the auditory pathway. BAER recordings were obtained at different stages from 2 control sheep and 22 other sheep fed high sulfur (S) diets with low or high concentration of thiamine (B_1), copper (Cu), and molybdenum (Mo). At least 4 peaks (I,II,III,IV) of varied amplitude were observed in all animals. Neurophysiologic abnormalities due to decreased conductivity and/or excitability of nerve fibers along the auditory pathway were found on the BAER recordings of sheep fed high S diet. Abnormalities of peaks and interpeak latencies within BAER recordings were related to histopathological observations of brain stem lesions. Lesions in the areas of the cochlear nuclei and lateral lemniscus were seen in conjunction with altered BAER components. However, abnormalities in BAER recordings and lesions in the brain stem occurred in the absence of overt clinical signs. Analysis of interpeak latencies between peaks I and IV revealed significant differences among dietary groups. Sheep given diets containing low (L) Cu-LMo-L B_1 were the worst affected. Factorial analysis indicated B_1 and interactions among

Cu, Mo, and B₁ as significant factors influencing interpeak latencies.

Impact and Significance for Saskatchewan Agriculture

A thiamine deficiency in cattle can affect brain function without major histological lesions occurring in the brain. These subtle effects of marginal thiamine status on performance of cattle and sheep indicate that animals may not respond normally to environmental stimuli following an occurrence of thiamine deficiency. Maternal traits in beef cows and effective responses to cold or hot environment could be affected by such a deficiency.

INTRODUCTION

Copper (Cu), sulfur (S), molybdenum (Mo), and thiamine (B₁) are considered essential nutrients for animals. However, these nutrients when imbalanced in the diet form a complex array of interactions, often leading to metabolic disturbances. Excess Mo and S in the diet decrease the availability of dietary Cu in ruminants (1). Recent reports have suggested a possible causal relationship between high S intakes, B₁ deficiency, and polioencephalomalacia (PEM) in cattle (2,3,4,5). Both B₁ (6) and Cu (7) are indispensable for brain metabolism. Hence a deficiency of either may lead to serious alterations of the normal function. Studies on early subclinical effects of essential nutrient deficiency on brain *in vivo* are few and the metabolic mechanisms of these deficiencies remains unclear.

Measurement of evoked potentials (EP) is a noninvasive, *in vivo* technique widely used in human medicine (8,12). One of the commonly used EP techniques is brain stem auditory evoked potential (BAER). It provides a noninvasive, reproducible and objective measure of brain stem function with a record of activity of the auditory pathway, reflecting electrical events ascending within the brain stem. BAER has been found to be extremely useful in the study of clinical neuropathology (8), particularly in the evaluation of neurologic disorders caused by demyelination, anoxic-ischemic encephalopathy and degeneration of neural tissue (8,9,10,11,12).

In recent years EP techniques have also gained an interest among veterinary investigators (13,14,15). Since it is a noninvasive technique it can be used to monitor the progress of brain lesions and/or response to treatment. In this study the BAER was used to evaluate the effects of high

dietary S on the brain physiology and compared with morphological abnormalities of the regions examined electrophysiologically. Histopathological examination of the areas was also undertaken.

MATERIALS AND METHODS

ANIMALS AND DIETS

Twenty-four, 2 month old cross-bred female sheep of body weights 12-15 kg were housed in individual metabolism units. The basal diet was a pelleted barley (59%) - soybean meal (5%) - alfalfa (32%) mixture prepared to meet the minimum nutrient requirements recommended by National Research Council (NRC) (16). The diet contained 0.63% of S with 2 levels of each of Cu, Mo, and B₁ (see footnote in Table 1). This diet was fed to 22 sheep. The remaining 2 sheep received normal S (0.19%) and 7.9, 4 and 234.0 mg kg⁻¹ DM of Cu, Mo and B₁ respectively. All animals had free access to water throughout the experiment and were offered the diets at rate of 1 kg per animal per day for 14 weeks.

BAER RECORDINGS

The BAER recordings were carried out on all sheep during the 14th week of experiment. Recordings from four (2 from diet 1, 1 from diet 2, 1 from diet 4) of the 22 high S fed animals were also obtained on the 8th week of the experiment. Prior to BAER recording, animals were injected with sodium pentobarbital intravenously (iv) (20 mg/kg body weight) to maintain them in a state of light anesthesia throughout the recording period. Sheep were positioned in sternal recumbency and testing was performed in a quiet room.

The BAER recordings were obtained from 3 platinum Grass E2B subdermal needle electrodes (Grass Instrument Co., Quincy, Mass). The active recording electrode was inserted subcutaneously just below the most ventral and caudal aspect of the pinna of the ear being tested and the reference electrode was likewise inserted at the vertex. The ground electrode was subcutaneously inserted in the untested ear, in a location similar to that of the active recording electrode. These recording electrodes were plugged into a SW-100A Electrode Selector (Nicolet Biomedical Instruments, Madison, WI) which was connected to a HGA-200A Physiological Amplifier (Nicolet Biomedical Instruments, Madison, WI) whose gain was set at 10^4 . The output of the HGA-200A amplifier was then fed into the Channels 1-2 input of the CA-1000 Clinical Averager. In recording the BAERs, the CA-1000 sensitivity was set at 10 ± 0.1 μ V and the low end of the input bandpass filter was at 30 Hz and the high end at 3000 Hz. The analysis time was 10 msec and the number of responses averaged was 2000.

Auditory stimulation.

Auditory stimulation was obtained from a Nic-10007A Click Stimulator/Noise Masking Module (Nicolet Biomedical Instruments, Madison, WI). The test stimulus consisted of a 100 microsecond condensation click of 90 dB intensity. This auditory stimulus was given at a rate of 11.1/sec and issued from a Nicolet bilateral insert earphone (Nicolet Biomedical Instruments, Madison, WI) that was securely placed within the ear. The untested ear received masking white noise at an intensity of 60 db.

HISTOPATHOLOGY

After the final BAER recordings, all sheep were euthanized with iv barbiturate and the brains removed for gross and microscopic analysis. Brains from 2 control sheep and 20 of 22 animals fed high S were examined for histopathological changes in the areas of the brain stem in relation to the electrophysiological events recorded on BAER. Brains were fixed in 10% buffered formalin and after fixation were sliced transversely to enable anatomical examination. Slices taken were routinely processed, embedded in paraffin, sectioned at 0.5 μ m and then stained with haematoxylin and eosin. Lesions were histologically graded; grade 1 - mild to moderate edema with mild gliosis and mild vascular endothelial swelling; grade 2 - severe edema with vacuolation of the neuropil, severe neuronal necrosis, moderate gliosis and severe vascular endothelial swelling; and grade 3 - total necrosis of all neural elements with glial cell formation and/or astrocytic scar formation. Care of animals was performed in accordance with the guidelines presented in the Guide to the Care and Use of Experimental Animals (17,18).

BLOOD THIAMINE

Blood B₁ concentration of all animals was monitored throughout the experiment using the biological method (*Ochromonas danica*) of Olkowski and Gooneratne (19) a modification of the method of Baker and Frank (20).

STATISTICS

Statistical analyses utilized General Linear Model Analysis of Variance (GLM ANOVA) from microcomputer package [Number Cruncher Statistical System (NCSS)] to determine treatment effects. Fisher's LSD (21) test was used for

mean comparisons among treatment groups.

RESULTS

None of the animals tested exhibited characteristic symptoms of PEM at the time of BAER recording. But 3 animals fed high S and no supplemental B₁ showed loss of appetite, mild depression and intermittent signs of excitation between 2 and 3 weeks after commencement of the experiment. These symptoms lasted only for 2 - 5 days and similar symptoms were not observed during the following 11 weeks prior to the BAER recording at week 14.

The normal pattern of BAER waveforms from a sheep on a low (L) S diet is shown in Fig. 1A. Waveforms, numbering of waves and anatomical relation of peaks were based on those published for horse (13) and human (9) since no data were available for sheep. A tentative scheme of anatomical-electrophysiological relationship was derived on assumption of similarity of the physiology among mammals (22). Anatomical differences such as sizes of nuclei and lengths of the fibers may exist in the brain stem of different species; however, electrophysiology is quite similar (22). All animals demonstrated at least 5 major positive peaks and up to 7 could be detected in some sheep (Fig. 1A). In sheep given high (H) S diets, abnormal pattern of BAER recording was observed with alterations in amplitude and latency periods and this was most marked in sheep which received either LB₁-LCu-LMo (Fig. 1B) or LB₁-HCu-LMo (Fig. 1C). Figure 1B shows the presence of all peaks but with altered amplitude and latency period. Figure 1C shows a BAER recording with severely depressed amplitude and increased latency. Recordings from animals

#23, 72, 130 fed high S - low B₁ diet with reference to respective schematic drawing of the damaged areas of the brain are shown in Fig. 2, 3, 4 respectively. Morphological damage was evident in all animals on this diet and consisted of edema, endothelial swelling of blood vessels, variable neuronal necrosis and gliosis. Figure 2 is a recording from lamb #23 fed HS-LCu-HMo-LB₁ with mild bilateral lesion in the area of lateral lemniscus. A noticeable decrement in the amplitudes of all BAER components were observed but on one side (Q₁) only, but latencies were increased on both sides (Q₁ and Q₂). Figure 3 depicts the effect of moderate to severe unilateral lesion of lateral lemniscus on BAER recording of sheep #72 which was fed HS-LB₁-LCu-HMo. But the amplitudes on both sides were markedly diminished. Figure 4 is a recording for sheep #130 (LB₁-HCu-LMo-HS) showing reduced amplitudes of all components of BAER on both sides (Q₁ and Q₂). This sheep had multiple necrotic lesions in the cochlear nuclei region. Figure 5 shows BAER recordings on the 8th and 14th week of the experiment from sheep # 229 fed HS-LCu-LMo-LB₁ diet and illustrates a progressive change in nerve conduction with time. A mild lesion of lateral lemniscus region was noted in this animal. The differences in wave patterns indicate the deteriorating effect of high S treatment on brain stem auditory pathway conduction.

Interpeak latencies (between peak I and IV) of BAER recording of sheep in different treatment groups are presented in Table 1. Significantly increased latencies ($p < 0.05$) indicative of reduced nerve conduction were seen in sheep not supplemented with B₁ (Groups 1, 2, 3, 4) in comparison to those receiving B₁. The most affected were the sheep on diets 1 and 4 (Table 1). The sheep from these 2 groups had greater latency periods than those on corresponding B₁ supplemented diets 1A and 4A. The latencies of sheep in the remaining two

groups which received supplemental B₁ (diet 2A and 3A) were not different to the latencies observed in sheep in corresponding groups not supplemented with B₁ (diets 2 and 3). Factorial analysis revealed the effect of supplemental dietary B₁ to be highly significant ($P < 0.0001$) in lowering the interpeak latency period regardless of the level of Cu or Mo in the diet. The interaction among Cu, Mo, and B₁ was also significant.

HISTOPATHOLOGY

Most animals fed a high S diet developed detectable lesions of PEM, with variable degrees of severity in the telencephalon, diencephalon, mesencephalon and metencephalon (Olkowski et al. unpublished results). No difference was noted in grade of lesions among sheep supplemented with B₁, except in the extra-pyramidal system. Two out of 8 animals which received B₁ supplementation had mild brain lesions (grade 1) in the cochlear nuclei or the lateral lemniscus region. Among the 12 sheep which did not receive supplemental B₁, 2 animals had grade 3 lesions and 3 animals had grade 1 lesions and grade 2 lesions in the rest in the aforementioned areas of the brain stem.

BLOOD THIAMINE

Blood B₁ concentration of control and test animals are shown in Fig. 6. The concentration of B₁ in control sheep and the test sheep which received B₁ supplementation (Cp 1A, 2A, 3A, 4A) were significantly higher ($p < 0.01$) than those test sheep which did not receive B₁ supplementation.

DISCUSSION

This study showed altered BAER measured auditory pathway nerve conduction in sheep fed high S diets and no supplemental B₁ compared with animals which received additional B₁ in the diet. This change in conduction was seen to co-exist with morphological changes along the neuronal tracts providing dual evidence for CNS damage following S supplementation. Alterations to BAER recordings have rarely been related with histopathologic lesions in domestic animals. The principal changes of BAER components observed in sheep given high S B₁-unsupplemented diets were 1) increases in interwave interval, 2) increases or decreases in amplitude, and 3) elimination of specific peak components.

Brain stem lesions have been shown in experimentally induced B₁ deficiency in dogs (23). Although sheep which did not receive supplemental B₁ were significantly more affected both in quantity and severity of BAER recordings, lesions in pertinent brain stem regions were not observed in all examined animals, indicating that BAER may be a more sensitive method of evaluating CNS damage in sheep. Changes in BAER pattern have been related to lesions in specific areas of the brain in humans (9,12,24), cats (25) and dogs (26). Although interpretation of such data in establishing pathogenesis of nervous disease is not yet clear it does indicate the possible use of BAER for ante-mortem diagnosis of CNS disease.

It is noteworthy that none of the animals tested exhibited characteristic symptoms of PEM at the time of BAER recording although observation of clinical signs was noted in some animals a few weeks before the readings were made. Therefore, changes in BAER waveforms may indicate a subclinical form of the

disease at the time of recording. Changes observed in recordings taken from the same animal at week 8 and 14 (Fig. 5) probably indicates the progressive development of brain lesion with time. Such serial recording and its correlation to a pathologic lesion, although it may not explain the mechanism of an abnormal BAER, may prove to be extremely useful in clinically assessing the progress of a patient. It is not known whether lesions were present at the time of BAER recording of sheep #229 during the 8th week of the experiment, but we speculate that changes in BAER may also occur in the absence of gross or histological lesions when an ultrastructural or a "metabolic lesion" is present, where the term metabolic lesion applies to an impairment of a vital enzyme or a coenzyme.

Blood B₁ concentration of B₁ non-supplemented sheep was significantly lower ($p < 0.01$) than in supplemented animals (Fig. 6). Prolonged interpeak latencies in sheep not supplemented with B₁ (Table 1) probably indicates metabolic distress that may have originated from metabolic lesions resulting from either toxic effects of S, S metabolites or S mediated B₁ deficiency. This study and the previously reported cases of PEM associated with high S intakes (2,3) is now presumed to be caused by a highly complex metabolic interactive effects of S with other minerals and vitamins (5).

Thiamine metabolism in the brain is unique. Brain appears to have regionally different requirements for B₁ (6). Chronic B₁ depletion has been associated with selective focal necrosis of the CNS (27). Thiamine deficiency may affect brain metabolism, by altering glucose and energy metabolism, and this may lead to a compromise of a variety of metabolic systems in the CNS. Myelin production can be impaired (6), and alteration in concentration of brain neurotransmitters has been reported (28,29,30). Thiamine is an integral

part of nerve membranes (31,32), and hence play a major role in nerve excitation and conduction. Since it is now known that high dietary S can cause B₁ depletion in ruminants (5), the changes in brain metabolism described above may possibly have resulted in prolonged interpeak latencies observed in the BAER recordings of the present study.

In conclusion, the evidence from this experiment indicates that high dietary S related B₁ deficiency is one of the key factors associated with abnormal BAER, although interactive effects of Cu and Mo with B₁ were also significant in inducing changes to latencies of BAER recordings. Further study is required to examine the metabolic pathogenesis of these abnormal neurophysiological findings.

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TABLE I Interpeak latencies (between I and IV) of brain stem auditory evoked response (BAER) recordings from sheep fed high sulfur (0.63%) varied levels of copper (Cu), molybdenum (Mo) and thiamine (B₁) and control sheep.

Diet No:	Diet description ^a	(n)	Interpeak latency mean $\pm$ SD (msec)
Control	(LCuLMoLB ₁ LS)	4	1.88 $\pm$ 0.1 ^d
1	(LCuLMoLB ₁ HS)	6	3.38 $\pm$ 0.2 ^b
2	(LCuHMoLB ₁ HS)	8	2.85 $\pm$ 0.18 ^{bc}
3	(HCuLMoLB ₁ HS)	8	2.88 $\pm$ 0.18 ^{bc}
4	(HCuHMoLB ₁ HS)	2	3.47 $\pm$ 0.2 ^b
1A	(LCuLMoHB ₁ HS)	2	1.80 $\pm$ 0.35 ^d
2A	(LCuHMoHB ₁ HS)	6	2.37 $\pm$ 0.2 ^{cd}
3A	(HCuLMoHB ₁ HS)	6	2.20 $\pm$ 0.2 ^c
4A	(HCuHMoHB ₁ HS)	6	2.47 $\pm$ 0.2 ^{cd}

ANOVA: Factorial analysis of dietary components.

Source of variation							
	B ₁	Cu	Mo	B ₁ xCu	B ₁ xMo	CuxMo	B ₁ xCuxMo
P	<0.0001	NS	NS	NS	NS	NS	<0.016

^a Diet description: Concentration of variable ingredients in the diets expressed as mean $\pm$ SD mg Kg⁻¹ DM. Low copper (LCu)=6.7 $\pm$ 1.1 Cu, High copper (HCu)=28.8 $\pm$ 1.1 Cu, Low molybdenum (LMo)=6.2 $\pm$ 1.8 Mo, High molybdenum (HMo)=22.5 $\pm$ 2.7 Mo, Low thiamin (LB₁)=13.7 $\pm$ 0.4 B₁, High thiamine (HB₁)=243 $\pm$ 22.2, LS=0.19% B₁.

n Refers to number of BAER recordings performed per treatment.

bcd Values with different superscripts within the column are different (p<0.05).

LEGEND FOR FIGURES

Figure 1: A. shows the pattern of BAER recording from a control animal fed low Cu-low Mo-low S-low B₁ diet. Several peaks identified as I,II,III,IV and V of differing amplitude are present.

B. pattern recorded from an animal (lamb #229) fed high S-low Cu-low Mo-low B₁ on the 8th week of the trial. Note increment of interpeak latencies.

C. A recording from an animal (lamb #186) fed high S-high Cu-low Mo-low B₁ diet for 14 weeks. A complete disappearance of all waves would indicate lesion at the beginning of the auditory pathway.

Figure 2: BAER recording (A) of lamb #23 fed high S-low Cu-high Mo-low B₁ diet. At post mortem a mild bilateral lesion (grade 1; ) in lateral lemniscus was seen (B).

Figure 3: BAER recording (A) of lamb #72 fed high S-low Cu-high Mo-low B₁ diet. At post mortem a severe (grade 3; ) to moderate (grade 2; ) unilateral lesion of lateral lemniscus was seen (B). Note significant decrement of amplitudes in recording in left and right ear (Q₁ and Q₂).

Figure 4: BAER recording (A) of lamb #130 fed high 2-high Cu-low Mo-low B₁ diet. At post mortem severe multiple lesions (grade 3; ) in cochlear nuclei region were seen (B).

Figure 5: A comparison of BAER recordings obtained from an animal fed high S-low Cu-low Mo-low B₁ diet on the 8th (A) and 14th (B) week. Note changes in

Q2 component with progression of feeding excess S. A mild lesion of lateral lemniscus was seen at post-mortem in this animal.

Figure 6: Blood thiamine concentration (g L^{-1} ; Mean $\pm$ SE) of 2 control sheep fed low S-low Cu-low Mo-high B_1 diet (☐) and 22 test sheep fed high S diet with (☒) (diet 1A, 2A, 3A, 4A; n=10) or without (☐) (diet 1, 2, 3, 4; n=12) B_1 supplementation. Diet description: 1 - $\text{LCuLMoLB}_1\text{HS}$; 1A - $\text{LCuLMoHB}_1\text{HS}$; 2 - $\text{LCuHMoLB}_1\text{HS}$; 2A - $\text{LCuHMoHB}_1\text{HS}$; 3 - $\text{HCuLMoLB}_1\text{HS}$; 3A - $\text{HCuLMoHB}_1\text{HS}$; 4 - $\text{HCuHMoLB}_1\text{HS}$; 4A - $\text{HCuHMoHB}_1\text{HS}$.

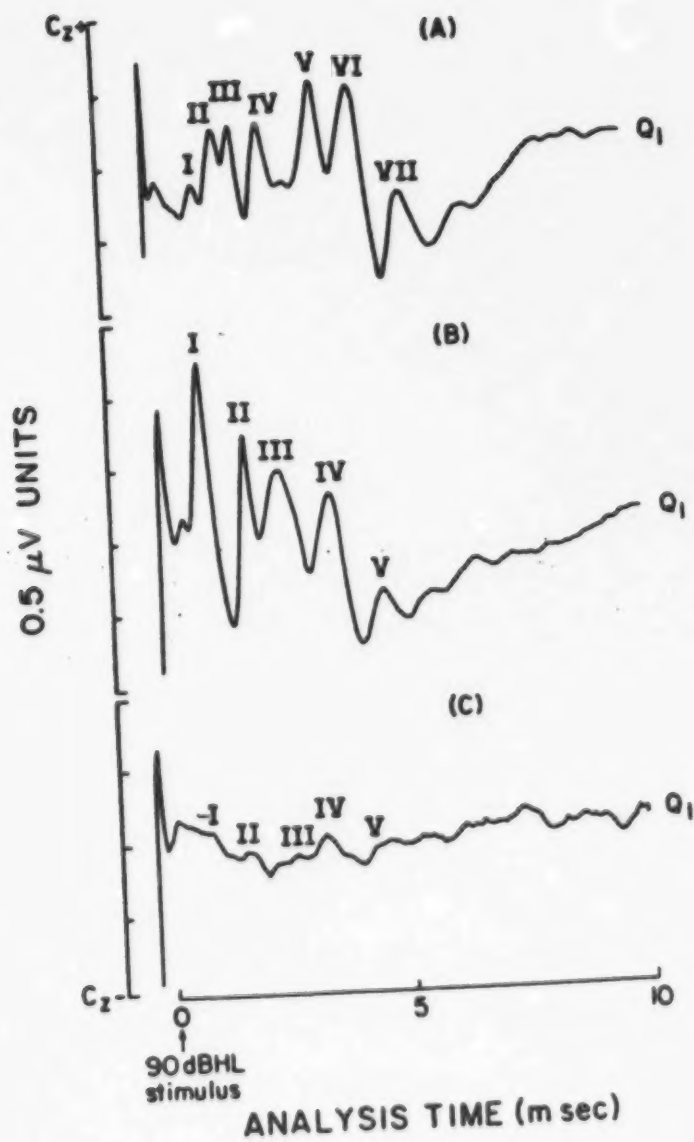
FIGURE 1

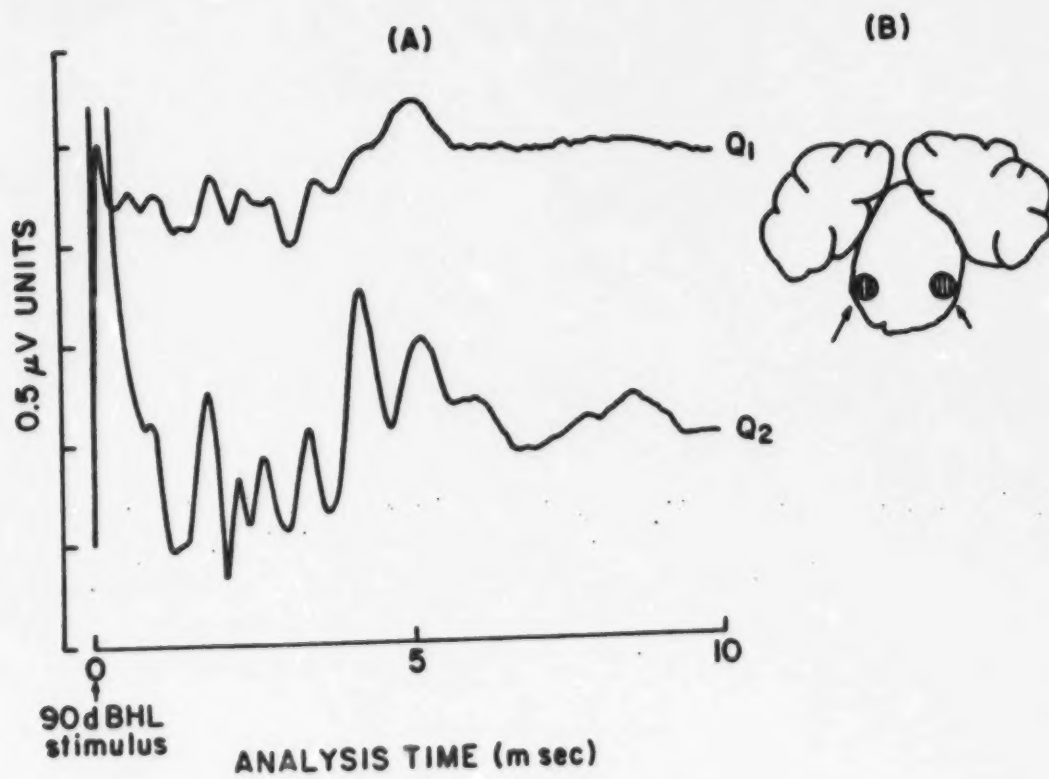
FIGURE 2

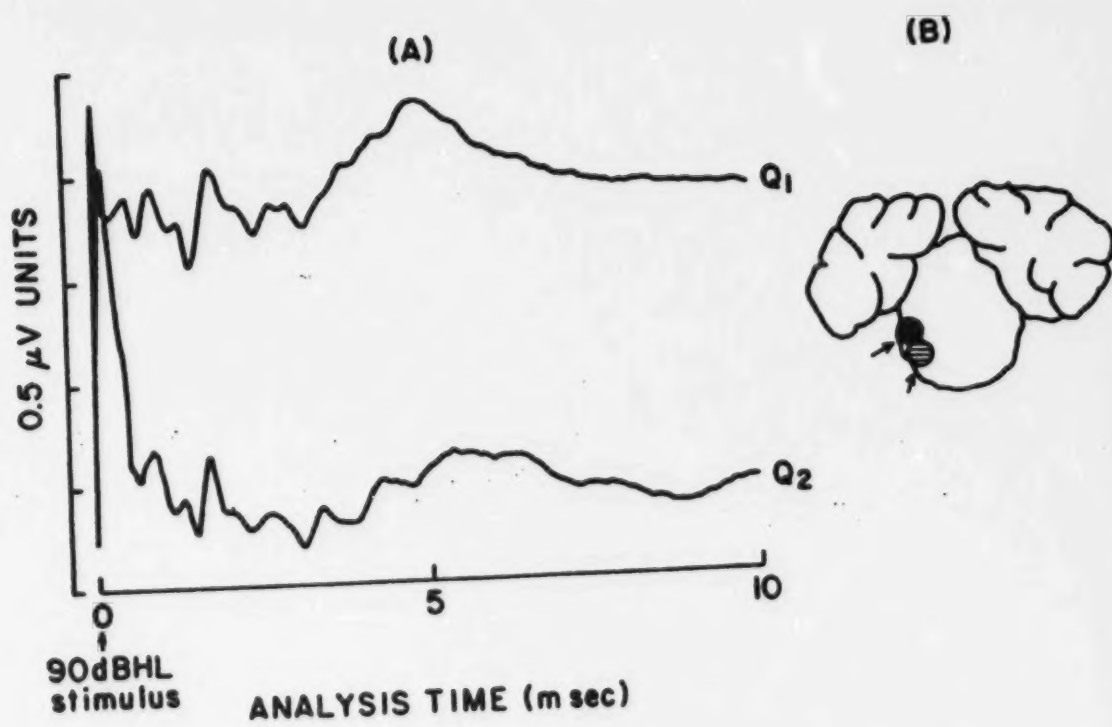
FIGURE 3

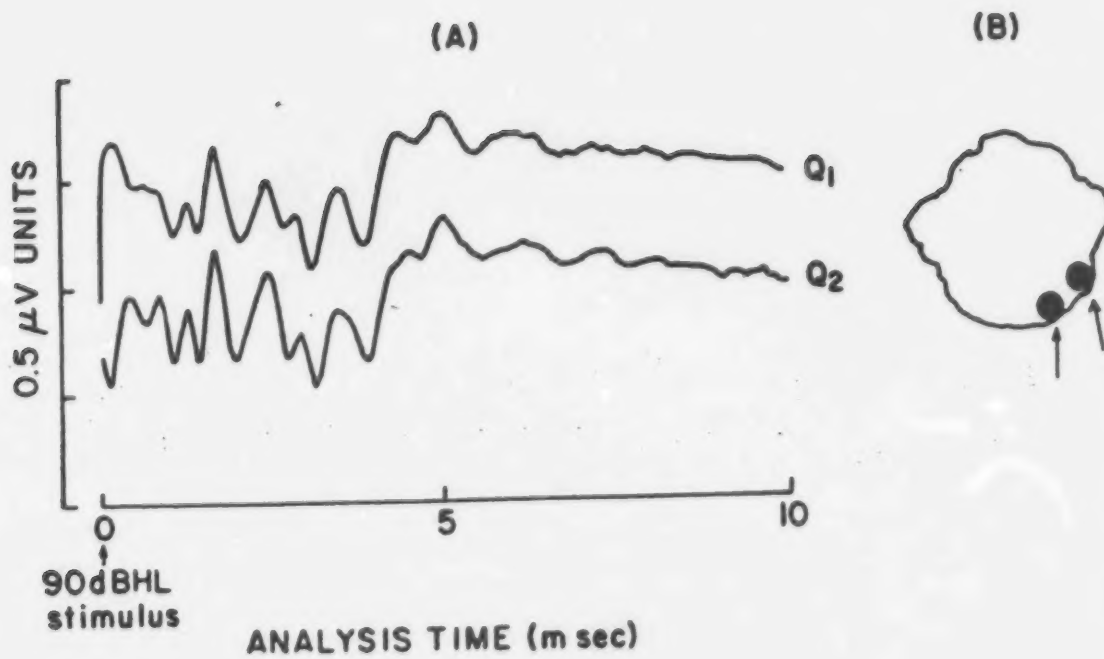
FIGURE 4

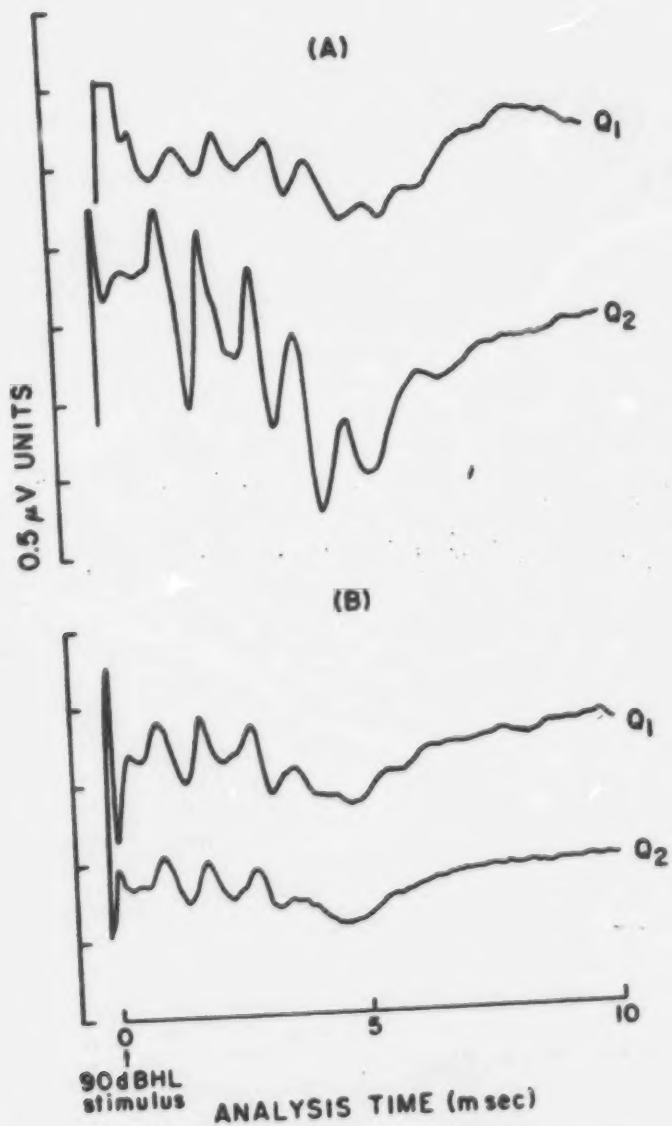
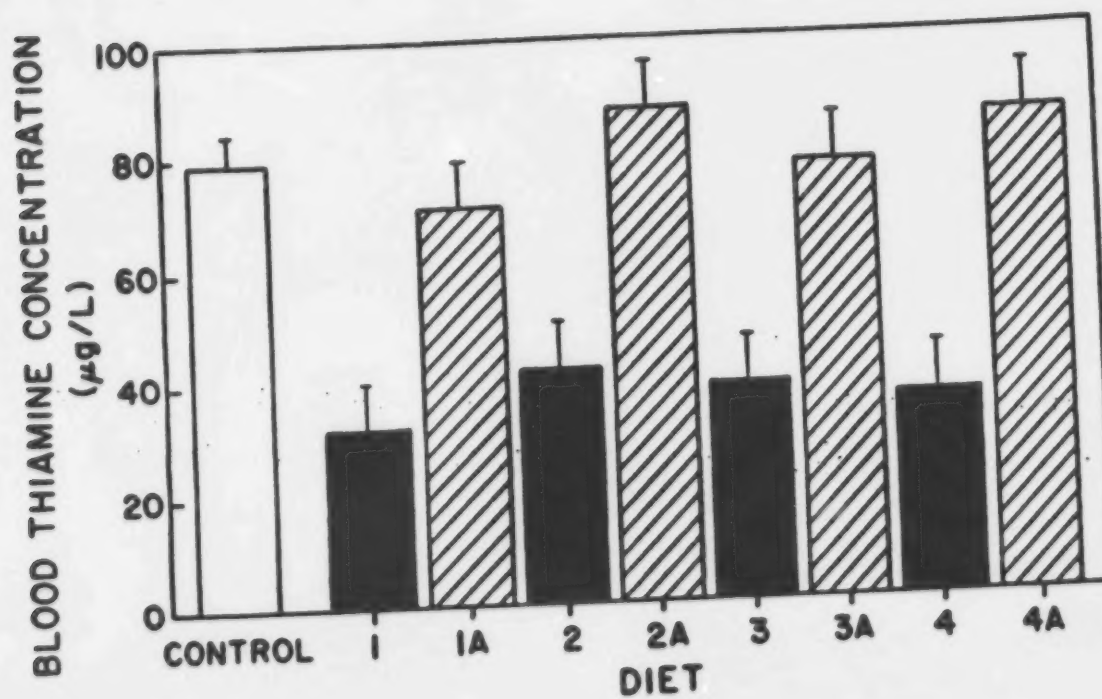
FIGURE 5

FIGURE 6

Part 3 Microbiological Methods of Thiamine Measurements in Biological Material Using 96-well Microplate Reader

A. Olkowski, R. Gooneratne and D.A. Christensen

Abstract

Semiautomated, microbiological methods for the determination of thiamine (B_1) were evaluated in bovine, ovine and rat blood, plasma, bile, urine, duodenal fluid, liver and brain. Thiamine was extracted from samples by mild acid digestion (trans aconitase pH 4.0) at 121°C for 10 min. Microbiologic assays utilizing Lactobacillus fermenti and Ochromonas danica were performed on 96 well microplates, and growth as monitored by optical density (OD) was directly read on a microplate reader at 650 nm. Particular attention was given to the validation of the two methods in terms of precision, accuracy, reproductibility, repeatability and recovery and technique comparison. *O. danica* method appeared to be a better indicator of biologically available B_1 , whereas the *L. fermenti* method under certain circumstances is prone to overestimation due to its inability to discriminate among metabolites or antimetabolites of B_1 .

Impact and Significance on Saskatchewan Agriculture

A reliable method for thiamine analysis was developed. Previous methods were slow and tended to overestimate the amount of biological active thiamine present in a feedstuff or in the ration. This improved method allows large numbers of thiamine assays to be conducted with improved accuracy of estimating the effective biological forms of thiamine.

3.1 Introduction

Thiamine (B₁) is an essential water soluble vitamin and thiamine pyrophosphate (TPP) is the most abundant form of the vitamin in the body. Thiamine pyrophosphate is required as a co-factor in a variety of biochemical pathways catalysed by transketolase, -ketoglutarate dehydrogenase and pyruvate dehydrogenase. Since these reactions are of central importance in ATP generation, a deficiency of this vitamin will particularly affect tissues with a high energy requirement such as nerves, heart and kidney. Thiamine deficiency manifests in ruminants as a neurologic disease known as cerebrotectal necrosis (CCN) or polioencephalomalacia (PEM).

Several biological methods for the measurement of thiamine (B₁), utilizing microorganisms, have been described. High performance liquid chromatography (HPLC) techniques for measurement of B₁ (Rindi and Giuseppe, 1961; Ishi et al, 1979; Botticher and Botticher, 1986) have been gaining popularity in recent years and are widely used in measurement of B₁ in food systems (Fingles and Faulks, 1987). However, due to the high cost of equipment and technical difficulties encountered in this technique, assays using the phytoflagellate Ochromonas danica (Baker and Frank, 1968) and

the bacterium, Lactobacillus fermenti (Sarett and Cheldelin, 1944) still appear to be the two most widely used procedures for estimation of B_1 . But the literature is scanty with respect to measurement of B_1 in biological material by these 2 methods not only in terms of reproducibility, repeatability and recovery data but by comparison with other procedures. Also there are misconceptions in the literature citing disadvantages of microbiological tests such as poor reproducibility, lack of automation, and time consumption (Botticher and Botticher, 1986). We have attempted to overcome these limitations by using a computer interfaced automated 96-well microplate reader to measure total and biologically available thiamine in body fluids and tissues using *O. danica* and *L. fermenti*. The phytoflagellata and bacterial methods described here are modifications of the assays of Baker and Frank (1968) and Sarett and Cheldelin (1944) respectively.

3.2 Materials and Methods

(a) Materials and procedures common for both methods

Trans-aconitate buffer: Trans-aconitic acid (Sigma) 5g, :
water 1000ml, (adjusted to pH 4.0 with KOH).

Thiamin Stock Solution: Thiamine hydrochloride (B_1 HCl) 1
mg/L in trans-aconitate buffer.

Reference sample: To establish a reference value, 1 L of
sheep plasma was stored as 1 ml allotments at -20°C .

Initially 25 assays were carried out (in quadruplicates) and the mean was taken as an internal reference value. This reference plasma standard was used with each assay to determine variations between assays.

Preparation of Standards: Standards containing 1.5, 3.0, 6, and 12 ug B_1HCl/L were prepared by diluting the thiamine stock solution in aconitate buffer and autoclaving at $121^{\circ}C$ for 10 min.

Preparation of samples: Samples of blood, plasma, bile, urine, tissues and feed were stored at $-20^{\circ}C$. Freshly thawed samples were added to aconitate buffer in 16x100 mm glass tubes at a ratio of aconitate buffer to blood/plasma/ bile at 1:4 v/v, to urine at 1:99 v/v, and to tissues at 1:10 v/w. Thiamine was extracted from tissues by homogenizing (Brinkmann PT 10/35 homogenizer, Brinkman Instruments, Rexdale, Ontario) in the buffer for 15 sec at max speed. All tubes were then vortexed, capped and autoclaved at $121^{\circ}C$ for 10 min. The homogenate was vortexed again while still hot and centrifuged at 2000 g for 10 min to obtain a clear supernatant containing B_1 . This B_1 extract was used for the assays. Some supernatants had to be further diluted in order to be within the linearity limits of the standard curve. Such supernatants were autoclaved again (after appropriate dilution) at $110^{\circ}C$ for 1 min. From this step onwards all equipment and materials used in the

assay were sterilized and all procedures were carried out using sterile techniques.

(b) Ochromonas danica assay

Stock culture of *O. danica* [American Type Tissue Collection (ATCC No.30004) ATCC laboratory Rockville, MA] was maintained on thioglycolate medium BBL-11260 (Microbiology Systems, Becton Dickinson, Cockeysville, MA) according to ATCC instruction. Three days prior to the assay, stock culture of *O. danica* was subcultured on BBL-11260 medium and transferred to an incubator illuminated with two 15-watt fluorescent lamps and a 60-watt incandescent lamp with temperature maintained at 30°C. A basic assay medium was prepared according to the method of Baker and Frank (1968) and the final assay medium was prepared by diluting the basic assay medium in distilled water at a ratio of 1.25:1. The *O. danica* inoculum was prepared by adding a few drops of 3 d old subculture to 5 ml of isotonic saline followed by vortexing and centrifugation at 1000 g for 5 min. The supernatant was discarded and the pellet containing the protozoa was next washed twice in saline followed by resuspension in saline (approximately 2 ml) at a concentration equivalent to approximately 0.7 optical density (OD) units at 650 nm wavelength.

The final assay solution was prepared by mixing 4 drops of the protozoal inoculum with 20 ml of final assay medium.

Aliquots (160 μ l) of this final assay solution was next added to each of the 96 wells in the microplate (Falcon 3792, Becton Dickinson Labware, Oxnard, CA) and allowed to remain at room temperature (RT) for 30 min. to condition the microplate prior to adding the samples. Equal volumes (40 μ l) of blank (aconitate buffer), standards, reference sample, and unknown samples were then added to the wells in the microplate in quadruplicates, followed by incubation at 30°C with illumination for 96 h. At the end of the incubation period, microplates were vortexed and read at 650 nm on the microplate reader (V max, Molecular Devices Corporation, Palo Alto, CA). All calculations were carried out instantaneously using a software program, 'SoftMax' (Molecular Devices Corporation, Palo Alto, CA) interfaced to the microplate reader.

(c) Lactobacillus fermenti method

Long term cultures of Lactobacillus fermenti (ATCC No. 9338, Difco Laboratories, Detroit, MI) were maintained as stab cultures of Lactobacilli agar (Difco Lab, Detroit, MI) at 2 - 8°C. Stock cultures were prepared by transferring stab culture to Lactobacilli broth (Difco Laboratories, Detroit, Michigan), and incubating at 37°C for 18 - 20 h. These cultures could be stored at 2 - 8°C for up to 2 weeks. Stock cultures were maintained by transfer to a Lactobacilli broth fortnightly. One day prior to the assay, a stock

culture of *L. fermenti* was transferred to Lactobacilli broth and incubated at 37°C for 16-18 hours. Bacterial inoculum was prepared by centrifuging the broth containing the culture at 2,000 g for 5 min. The supernatant was discarded, and the remaining pellet containing bacteria was washed three times in isotonic saline and resuspended in saline at a concentration approximately equivalent to 0.7 OD units at 650 nm. The final assay medium was prepared by mixing 8.5 g of dehydrated Lacto thiamin assay medium (Difco Lab, Detroit, MI) with 160 ml of distilled water and boiling for 3 min. This was distributed into 20 ml allotments and sterilized at 110°C for 1 min. The final assay solution was prepared by mixing 4 drops of bacterial inoculum with 20 ml of final assay medium. Aliquots (160 ul) of this solution was next added to each of the 96 wells in the microplate 30 min prior to adding the samples. Equal volumes (40 ul) of blank (aconitate buffer), standards, reference sample, and unknown samples were then added into the wells of the microplate in quadruplicates, followed by incubation at 37°C for 16-18 h. Following this, the microplates were vortexed and read directly on the microplate reader at 650 nm. All calculations were carried out instantaneously using a software program, 'SoftMax', interfaced to the microplate reader.

3.2.1 Statistical analysis

Statistical analyses were carried out using General Linear Model Analysis of Variance (GLM ANOVA) from microcomputer package [Number Cruncher Statistical System (NCSS)]. Fisher's LSD test was used for mean comparisons of B_1 concentrations of body fluids among different species.

3.3 Results

Since there is no unanimity in the literature as to units of expression of thiamine concentration in samples, all thiamine values presented in this manuscript are expressed in terms of B_1HCl . To convert these values to concentration of thiamine, a conversion factor of 0.7868 needs to be used to express on a weight basis and a conversion factor of 2.9649 to express on molar basis. Typical standard curves for *L. fermenti* and *O. danica* are shown in Figs. 3.1 and 3.2 respectively. Lowest detection limit for both methods was $0.5 \text{ ug}B_1HCl/L$. Correlation coefficient of the standard curve obtained from either assay was > 0.999 . With both methods, linearity of the standard curve was maintained up to $2.4 \text{ ug}B_1HCl/L$ which is equivalent to $12 \text{ ug}B_1HCl/L$ in the original sample since the dilution in the microplate was 5 fold. Table 3.1 shows B_1 concentration in a wide variety of biological samples from cattle, sheep, and rat as assayed by the *L. fermenti* method. Concentration of B_1HCl in rat plasma was approximately 3 times higher than that of cattle ($p < 0.01$) and sheep blood concentrations, but the B_1HCl concentration

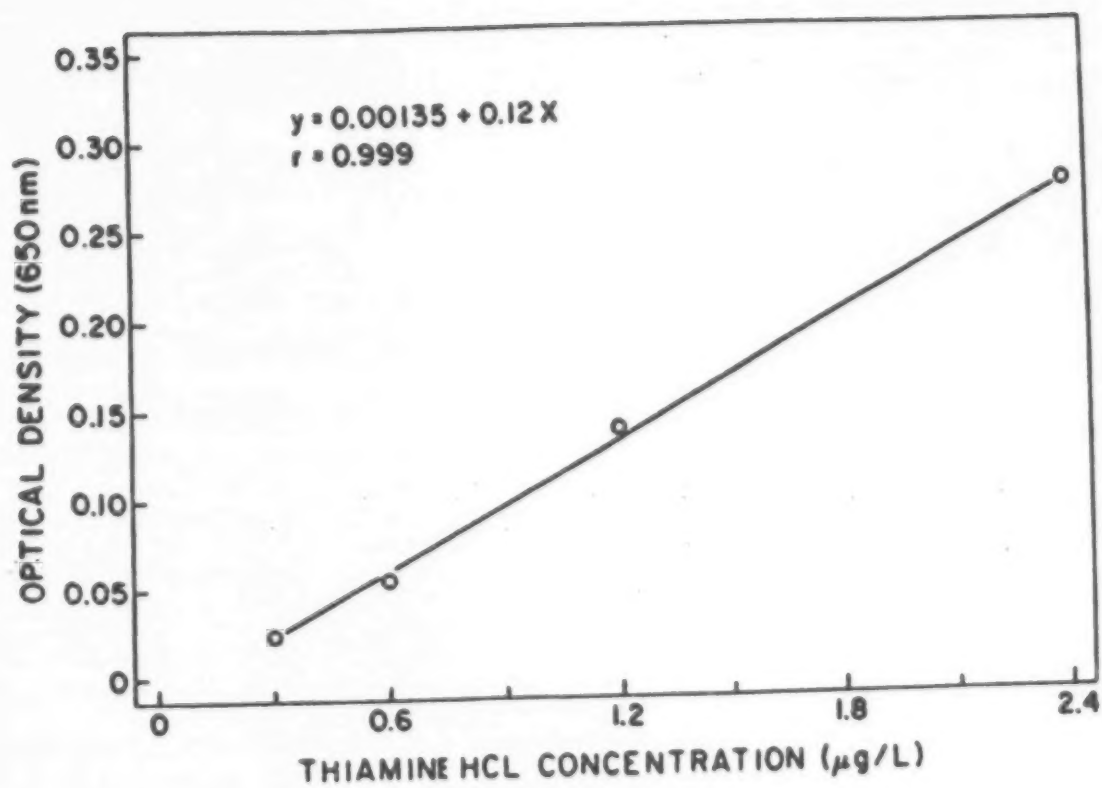


Figure 31 A typical standard curve for estimation of thiamine with *Lactobacillus fermenti* method.

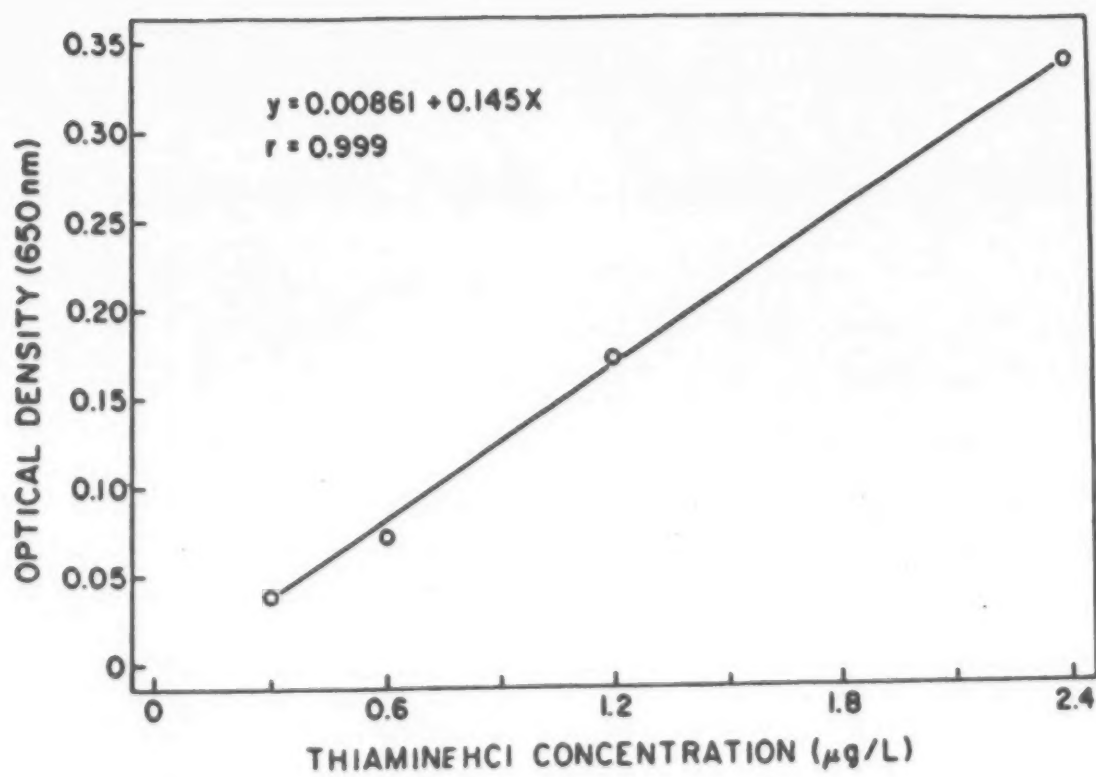


Figure 32 A typical standard curve for estimation of thiamine with *Ochromonas danica* method.

TABLE 3.1

Thiamine concentration in normal biological material from cattle, sheep and rats as assayed by the *L. fermenti* method.

Sample and species	Thiamine concentration *		
	mean	SD	range
Whole blood (ug/L)			
- cattle	78.8	22.32	50 - 112
- sheep	85.2	12.98	68 - 103
Plasma (ug/L)			
- rat	268.8	37.25	212 - 320
Urine (ug/L)			
- sheep	2764.2	1825.99	569 - 4775
- cattle	194.2	94.47	101 - 291
Bile (ug/L)			
- sheep	42.46	16.18	21.5 - 67.5
- cattle	11.2	4.07	5.6 - 15.2
Duodenal fluid (ug/L)			
- sheep	1.15	0.54	0.7 - 2.5
Sheep tissues (mg/Kg)			
- liver	7.5	1.68	5.4 - 9.3
- kidney (cortex)	4.1	0.75	3.2 - 5.5
- brain (grey matter)	1.1	0.15	0.96 - 1.37
- heart	4.6	0.76	3.6 - 5.5
Rat tissues (mg/kg)			
- liver	9.1	4.06	5.4 - 15.9
- brain (whole)	4.4	1.36	2.8 - 6.6

* Refers to mean, standard deviation (SD), and range for 6 samples for each category of each species.

of rat liver and sheep were similar. Concentration of B_1HCl in urine and bile of sheep was several times higher ($p < 0.01$) than that of cattle. A comparison of the 2 methods (Table 3.2) shows that the result of both *O. danica* and *L. fermenti* methods are comparable for blood, brain, and plasma of sheep. Recovery assays carried out by adding known amounts of B_1HCl to blood, plasma, urine, and liver homogenate of sheep using the *L. fermenti* method are shown in Table 3.3. Recoveries ranged from 93% to 110%. *L. fermenti* assays carried out to determine the optimum ratio of plasma to buffer showed that plasma to buffer ratio between 1:2 and 1:4 had no effect on recovery of B_1 (Table 3.4). Assays performed with *L. fermenti* on microplates were compared to the more traditional method of using glass tubes (Table 3.5). Both the recovery and coefficient of variations were similar.

3.4 Discussion

The present study shows for the first time that the microbiological assays of B_1 could be performed on microplates with no loss of sensitivity or precision. Among the variety of procedures reported in the literature for measuring thiamine concentration in biological material, the two methods (bacterial and protozoal) reported in this study were selected due to the following reasons. The thiochrome assay, one of the oldest B_1 assay systems (Hennessy & Cerecedo, 1939; Birch et al, 1952), depends on the oxidation

TABLE 3.2

Comparison of the two microbiological methods (*O. danica* and *L. fermenti*) in measurement of thiamine content in sheep blood, plasma (ug/L), and brain (mg/kg).

Sample *	<i>L. fermenti</i>		<i>O. danica</i>	
	Mean	cv ¹	Mean	cv ¹
Blood 1)	111.95	2.2	103.50	2.3
2)	114.65	3.1	109.90	4.5
3)	87.70	4.7	86.80	3.7
Brain 1)	1.20	5.6	1.29	2.5
2)	0.98	6.8	0.97	7.1
3)	1.04	4.3	1.06	4.7
Plasma (reference)	21.80	5.8	21.65	6.8

* 3 samples each of blood, brain and plasma reference were assayed in quadruplicates.

¹ CV refers to coefficient variation (variation between quadruplicates).

TABLE 3.3

Results of thiamine (B₁) recovery tests * using *Lactobacillus fermenti* method.

Sample	Thiamine (ug/L)			CV ¹	% Recovery
	Original Concentration	Amount Added	Final Concentration		
Plasma	21.35	20.0	43.3	4.8	110
	21.35	40.0	63.7	3.8	106
	21.35	60.0	79.9	3.1	98
Blood	59.88	20.0	80.8	5.1	105
	59.88	40.0	97.2	4.6	93
	59.88	60.0	122.0	2.1	104
Urine	1935.00	1000.0	2987.0	4.1	105
	1935.00	1500.0	3486.0	4.5	103
	1935.00	2000.0	4008.0	3.2	104
Liver homogenate	1457.00	300.0	1743.0	6.8	95
	1457.00	400.0	1864.0	5.3	102
	1457.00	500.0	1953.0	2.4	99

* Known amount of B₁ hydrochloride was added to either plasma, blood, urine or liver homogenate and the % of recovered B₁ calculated using the *L. fermenti* assay. % recovery was calculated using formulae: (final conc. - original conc) x 100/amount added.

¹ CV refers to coefficient variation (variation between quadruplicates).

TABLE 3.4

Evaluation of varied ratios of sample (plasma) to buffer on reproducibility of the L.fermenti method.

Ratio (Sample : Buffer)	Thiamine concentration	
	(ug/L)	cv ¹
1:2	21.78	4.5
1:3	21.6	6.4
1:4	21.8	5.2

¹ Cv refers to coefficient variation (variation between quadruplicates).

TABLE 3.5

Comparison * of *L. fermenti* assay performed in glass tubes and in microplates.

Blood Sample No.	Glass Tube		Microplate	
	(ug/L)	CV ¹	(ug/L)	CV ¹
1	76.18	2.4	75.72	2.7
2	29.57	6.3	29.04	4.6
3	46.34	4.5	46.2	3.7
4	37.46	3.5	36.12	4.5

* Blood samples were assayed in conventional glass tubes and microplates using *L. fermenti* method.

¹ CV refers to coefficient variation (variation between quadruplicates).

of B_1 by ferricyanade into thiochrome which is measured fluorometrically. The main drawbacks of this method are (i) differences in fluorescence of B_1 metabolites (Penttinen, 1976), and, (ii) interference by non- B_1 natural fluorphores similar to thiochrome (Loew et al, 1972), which are often present in biological material (Loew & Dunlop, 1972). Therefore, the use of the thiochrome method in measurement of B_1 concentration in biological material is prone to error and hence questionable.

Determinations of B_1 by HPLC methods (Rindi & Giuseppe, 1961; Ishi et al, 1979; Botticher & Botticher, 1986; Finglas & Faulks, 1987) appear to be both sensitive and accurate. However, since the method is based on the thiochrome reaction, drawbacks outlined above, may influence the results unless sophisticated purification procedures are employed. Hence HPLC methods have a more suitable application in pure science rather than in applied science such as in biological systems. Overestimation of B_1 may also occur with HPLC method since the presence of B_1 metabolites, biologically inactive B_1 , or substances potentially affecting bioavailability of B_1 are not taken into consideration in evaluation of B_1 concentration of the samples. This is an important consideration in biological systems since the presence of B_1 in a biological sample at a level recognized as adequate does not necessarily mean that this B_1 is completely available to the organism. Therefore, from a

practical point of view, evaluation of B₁ status of mammals for nutritional purposes using microbiological methods have definite advantages over the other methods described in the literature. The microbiological methods are as sensitive as the HPLC based assay systems. They are also extremely specific, less expensive, and more importantly, require less sophisticated instrumentation and hence could be set up in many laboratories. The common disadvantages most often emphasized in the literature concerning microbiologic methods are poor reproducibility and lack of automation (Botticher and Botticher, 1986). Our study shows that both these factors can be overcome by using the microplate based assay procedure. A number of factors can affect reproducibility of an assay, namely improper technique, use of biologically impure strains for assays, and contaminated or non defined media. Experiments described here indicate that both *Lactobacillus* and *Ochromonas* methods if carried out properly can ensure good reproducibility, specificity and sensitivity. Our attempt at partial automation by culturing assays on 96 well microplates and using a multichannel pipette for transfer of media to the microplates, allowed us to analyse small sample sizes (1 ul - 200 ul) and increase efficiency by reducing the time spent for each assay. Use of a microplate reader equipped with a computer interface allowed us to further reduce time and enabled us to read and calculate data from 96 wells (quadruplicates of 18 samples, 1 blank, and 5

standards) in less than 1 minute. Currently, 96 well microplate readers are routinely used in many immunological procedures and have recently been found to be extremely useful for measuring enzyme activities (Ashour et al, 1987). The use of a 96 well microplate for routine microbiological B₁ assays allows processing of a large number of samples rapidly, efficiently and at a low cost. The results presented in table 2 indicate a high degree of agreement between protozoal and bacterial methods of B₁ estimation. However, in analyzing blood, bile, and urine from animals exhibiting clinical symptoms of B₁ deficiency, the protozoal assay detected 2 to 5 times less B₁ than with the bacterial assay (Olkowski and Gooneratne, unpublished results). The reason for such a finding is not clear but we presume that bacteria may be less demanding in terms of absolute requirements for B₁ and, hence in conditions of non-availability of B₁, the bacteria would still be capable of utilizing even the breakdown moieties of B₁. Bacteria thus appear to be able to recognize a variety of breakdown products of B₁ including metabolites of B₁, or be less affected by potential factors obstructing bio-availability of B₁. By contrast, *O.danica* which is considered a model microorganism, expressing a mammalian-like requirement for B₁, is probably capable of utilizing only the biologically available form of B₁. Hence the major difference between the two assay systems appear to be the ability of *L.fermenti* to

recognize even metabolites or antimetabolites of B_1 in samples which the *O. danica* seems to be incapable utilizing, due to either interference with uptake and/or metabolism of B_1 . Our studies (Olkowski and Gooneratne, unpublished results) have shown that this growth inhibition of *O. danica* by B_1 metabolites can in fact be overcome by addition of an excess of B_1HCl to the sample. This provides evidence that biologically available B_1 was the limiting factor. But the extent of recognition of different forms of B_1 in biological material by bacteria and protozoa requires further investigation. Our studies indicate that the protozoal method is a better indicator of biologically available B_1 whereas the bacterial method under certain circumstances is prone to overestimation due to its apparent ability to recognize even metabolites/antimetabolites of B_1 .

In conclusion, the 2 microbiological methods of B_1 measurement presented here are simple, can be semi-automated, have a low detection limit and a high degree of precision and reproducibility. Either assay (*L. fermenti* or *O. danica*) could be used to detect a B_1 deficiency state. But when both assays are performed on the same samples, a comparison of the results from the bacterial and protozoan methods would enable one to distinguish whether B_1 deficiency was caused by a primary deficiency of B_1 per se, or was related to the presence of B_1 anti-metabolites.

Part 4 Lead Toxicity in Ruminants; Therapeutic Potential of Thiamine. The Effects on the Metabolism of Copper, Zinc, Iron and Manganese.

A. Olkowski, R. Gooneratne and D.A. Christensen

Abstract

Animals were surgically modified to allow collection of bile without interfering with physiological processes. The effect of thiamine on chelation therapy with EDTA and biliary and urinary excretion of lead was studied on cattle and sheep. The effect of repeated injections of thiamine on tissue residue of lead was investigated using sheep chronically exposed to lead in the diet.

Sheep chronically exposed to lead and depressed blood thiamine. Increased thiamine excretion upon intraduodenal infusion of lead was observed both in bile and urine. Subcutaneous administration of thiamine (75mg/kg) resulted in elevated blood lead content and increased biliary and urinary evacuation of lead. The efficacy of chelation therapy with EDTA was enhanced by co-treatment with thiamine. Repeated injections of thiamine (50 mg/kg) once a week for five weeks decreased lead content in bone and brain, but increased lead content in spleen and kidney.

Impact and Significance on Saskatchewan Agriculture

Farm livestock may be exposed to potential lead toxicity from a number of sources. The usual treatment is to administer a compound which will form a stable complex with lead and which is excreted in the bile or urine. Lead toxicity causes a reduction in plasma, thiamine level and so it was suspected that in addition to the possibility of lead toxicity causing secondary thiamine deficiency, that the administration of thiamine might assist in secretion of lead. In this experiment conducted with surgically altered sheep it was found that thiamine did decrease lead content of bone and brain. It

was concluded that an intake of adequate amounts of thiamine may have a long term beneficial effect in reducing accumulation of lead, but there is little benefit of short term administration of large amounts of thiamine in alleviating acute lead toxicity.

4.1 Introduction

Lead poisoning remains one of the most often occurring toxicosis in cattle. The frequent occurrence of this problem can be partially attributed to certain behavioral and anatomo-physiological characteristics of cattle. Natural curiosity and indiscriminate eating habits make this species particularly vulnerable to ingestion of lead-containing objects. Also nutritional errors leading to pica may be a predisposing factor. Once ingested, lead may be retained in the reticulum over long periods of time, allowing gradual absorption and intoxication. The main sources of lead in the farm environment are lead-containing petroleum products, batteries, plumbing lead, lead lined tanks, lead based paints, etc. (Buck et al 1982). Young animals are more susceptible to lead toxicity (Buck et al 1982, Blakley 1984). The characteristics of lead poisoning in cattle are greatly influenced by agricultural activities, with a predominance of cases occurring during summer months (Blakley 1984). A single dose ranging from 400-600 mg/kg in calves and 600-800 mg/kg in adult cattle is lethal (Buck et al 1982). Current therapeutical methods of Pb poisoning are mainly based on artificial chelating agents. EDTA is the most recommended

drug for treatment because it is relatively more effective and less toxic than any other chelating agent. However, numerous shortcomings of EDTA therapy and the inherent toxicity of this drug make its use not completely effective or desirable (Bratton et al 1981a,b). The main route of excretion of Pb-EDTA complexes is via urine, yet Pb is known to cause renal damage. The kidneys are of primary importance in managing, not only excretion of toxic metals, but also the homeostasis of essential metals. The changes in metabolism of essential trace elements can be due to direct interactions with lead and the deleterious effects of lead on renal function. Chelation therapy can affect homeostasis of essential metals. Victory et.al (1986) demonstrated increased excretion of copper, zinc, and, iron during chelation therapy with EDTA in rats. The urinary excretion of zinc, iron, and manganese in mice was increased by EDTA (Cantilena and Klaassen 1982).

In the search for a compound which has low inherent toxicity and effectively reduces lead toxicity, thiamine was selected as having promising potential in prevention and treatment of lead poisoning. Several authors indicated the beneficial effects of thiamine. Bratton et. al (1981a,b) demonstrated in calves that thiamine in therapeutic doses administered simultaneously with lead reduced lead toxicity and prevented deposition of lead in tissues. Similar findings were reported by Flora and Sharma (1986) from

experiments on rats. Exposure to lead in thiamine pretreated rats caused increased absorption and uptake of lead by tissues initially, followed by rapid release of lead from tissues (Sasser et.al 1984). These findings indicate that thiamine plays an important role in lead kinetics in the body. Ito et.al (1987) observed that lead excretion in feces and urine was enhanced by injection of thiamine. The same authors hypothesized that administration of thiamine may activate the transport mechanism of biliary excretion of lead. The excretion of lead in bile was shown to be one of the major routes of elimination of lead from the body (Blaxter and Cowie 1964, Castellino et.al 1966). The combination treatment of artificial chelating agents and thiamine appears to be more effective than the single effect of either. Thiamine significantly enhanced the effect of EDTA on urinary excretion of lead (Flora et.al 1985, Kachru et.al 1987) and decreased blood, liver, and kidney lead concentration (Kim 1988). Combination therapy appears to be effective in reducing tissue concentration of lead when agents were administered simultaneously with lead (Flora et.al 1985), but only marginally influenced mobilization of tissue lead from lead loaded rats (Kachru et.al 1987).

4.2 Blood concentration and biliary and urinary excretion of lead, copper, zinc, iron, and manganese in cattle after intraduodenal administration of lead acetate: The

effects prior to and after challenge with thiamine, EDTA, and a combination of thiamine and EDTA.

4.2.1 Materials and methods

4.2.1.1 Animals and diet

Three pure breed Simmental cows approximately 4 years of age were used in the experiment. Cows were housed in individual pens. Each cow was offered a pelleted ration of 8 kg/day in two equal portions at 9:00 a.m. and 4:00 p.m. and tap water ad libitum. The diets were prepared to meet nutritional requirements for beef cattle (NRC-NAS). For composition and mineral content of the diet see Appendix A (Table 1). Animals were surgically modified to allow collecting samples of bile and measuring bile flow without interfering with enterohepatic circulation and normal digestive processes.

4.2.1.1.1 Surgical procedure

The modification of the proximal duodenum to allow collection of bile in cattle was performed as described by Symonds et al (1982). Briefly: The animals were starved for 24 hours prior to induction of general anesthesia and the surgical site was clipped and cleaned. Following induction the animals were placed in left lateral recumbency and the surgical site was prepared for surgery. Full aseptic

surgical protocol was followed for the surgery. An 18 inch paracostal incision was made through the skin and underlying muscle layers. The duodenum was identified and after placing bowel clamps, was transected proximal to the point of entry of the common bile duct. A second set of clamps were placed distal to the point of entry of the bile duct and the gut transected at this point as well. The continuity of the intestinal tract was re-established by anastomosing the two segments of the duodenum using a simple end-to-end technique with 2-0 polyglycolic suture. The proximal end of the isolated portion of the duodenum was closed with a Parker-Kerr oversew to form a liquid tight seal. The distal end of the isolated portion was secured to a plastic cannula with a simple purse string suture and the cannula exited through the body wall on the proximal anterior aspect of the skin incision. A T-shaped cannula was exited through the body wall just ventral and caudal to the egress cannula and this was inserted into the re-anastomosed duodenum to allow bile to flow back into the intestinal tract. The T cannula was inserted into the antimesenteric aspect of the duodenum and was secured with a purse string suture. Following routine closure of the body wall, both cannula were secured in place with adhesive tape ensuring there was firm contact between the peritoneum and portions of bowel into which the cannula entered. This was considered important to ensure good fibrosis between these structures to prevent contamination of

the abdomen with either bile or intestinal contents. The two cannulae were connected externally with a plastic tubing containing a one way valve system.

4.2.1.2 Experimental Design

Lead was administered as 3% aqueous solution of lead acetate (Fisher) containing 16.81 mg/ml of Pb. Each cow received a dose of 12.5 mg/kg/day of lead through duodenal cannula. The daily dose was administered in two equal portions at 9:00 a.m. and 4:00 p.m. The trial was carried out according to the schedule shown in table 4.2.1. The EDTA (Leadidate, MTC-Pharmaceuticals, Mississauga, ON) was administered at the rate of 66 mg/kg BW through intravenous cannula. Thiamine HCl (Rafter 8 Products, Calgary, AB) was administered subcutaneously at the rate of 10 mg/kg BW.

4.2.1.3 Sampling schedule

Samples of blood, bile and urine were collected from each cow. An identical sampling schedule was established for each trial. For analysis, samples of all aforementioned specimens were collected for 1 hour prior to treatment and every one hour after treatment for 5 hours.

4.2.1.4 Sample collection

4.2.1.4.1 Urine

TABLE 4.2.1 Schematic summary of experimental design.

Day of Exp.	Pb Infusion	Treatment	Sampling	Trial
1	yes	non	no	
2	yes	non	no	
3	--	single dose Pb 12.5 mg/kg	yes	A
4	no	thiamine	yes	B
5	yes	non.	no	
6	yes	non	no	
8	no	EDTA	yes	C
9	yes	non	no	
10	yes	non	no	
12	no	EDTA + thiamine	yes	D

On the day of sampling cows were catheterized into the bladder with inflatable Foley catheters 20 Fr/30cc (Inmed Corporation, Nercross, Georgia). Urine was collected into plastic bags. Output volume was recorded every hour and a sample (approx. 6 ml) was collected into plastic tubes. The tubes were stoppered and stored at -20°C .

4.2.1.4.2 Bile

Bile was collected from bile cannula into a plastic bag for 30 min. Output volume was recorded and a sample (approx. 3 ml) was collected. Next, the remaining bile was immediately returned to the duodenum. Two 30 minute samples from each hour of sampling were pooled in proportions corresponding to respective output volumes into one plastic tube. The tube was covered with a stopper. All such obtained 1 hour samples were stored at -20°C . for further analysis.

4.2.1.4.3 Blood

Blood was obtained from the jugular vein through intravenous cannulae. Samples (5 ml) were collected into plastic tubes containing 60 I.U. of heparin. Tubes were covered with plastic stoppers and stored at -20°C . for further analysis.

4.2.1.5 Measurements

4.2.1.5.1 Clinical features

Clinical appearance and vital neurological signs were monitored twice every day throughout the experiment.

4.2.1.5.2 Analytical measurements

Analysis of thiamine was performed as described in section 3.0.

For mineral analysis samples were digested in a mixture of nitric/perchloric acids (3.5:1.5 v/v). Copper, zinc, iron, and manganese were measured on an atomic absorption spectrophotometer (Perkin Elmer, model 5000). Lead analysis was performed on a Perkin-Elmer Model 2280 atomic absorption spectrophotometer with deuterium-arc background correction and a HGA-500 electrical atomizer. Pyrolytic graphite coated tubes and platforms (Perkin-Elmer) were used. The spectrometer was interfaced with an IBM-PC microcomputer to enable rapid simultaneous monitoring of absorbance and background correction. Absorbance measurements were made at 283.3 nm. A phosphate as $\text{NH}_4\text{H}_2\text{PO}_4$ was used as matrix modifier (Eaton and Holcombe 1983, Hinds and Jackson 1987).

4.2.1.6 Analysis of Data

Statistical analysis were carried out using General Linear Models (GLM ANOVA) from microcomputer package [Number Cruncher Statistical System (NCSS)]. Fisher LSD test

(Snedecor and Cochran 1980) was used to determine statistically significant differences between the means. Figures were prepared with the SAS program and MacIntosh Apple computer using the program "Cricket".

4.2.1.7 Basal level of excretion

The samples collected for one hour before each treatment provided average values for basal level of excretion. It was assumed that each animal would serve as its own control and the average value of samples collected on different days would account for day to day variability.

4.2.2 Results

4.2.2.1 Clinical features

Shortly after intraduodenal administration of lead all animals showed symptoms of toxicity. Signs of excitation appeared first and were followed by profuse salivation. Hypersensitivity to voice and touch expressed by rapid blinking of eyelids, violent movement, shaking of head, and vocalization were seen in one animal. Bile flow was decreased to less than 10% of normal in all animals. Frequent urination was observed, however urine output was considerably smaller. The bile flow returned to normal after approximately 4 hours, and all animals appeared to be normal within the 5th hour after lead administration. Decreased

appetite was observed in all cows throughout the experiment.

4.2.2.2 Analytical data

4.2.2.2.1 Changes in blood lead concentration and biliary and urinary excretion of lead after intraduodenal administration of lead acetate in cattle.

Lead administered intraduodenally as acetate salt was absorbed rapidly into the blood stream. Absorption appeared to reach its plateau approximately 5 hours after infusion. During this time blood lead concentration increased 4 times as compared to the level before treatment. Biliary lead excretion was decreased during the first three hours after treatment. This decrease was inadvertently related to a substantial reduction in bile flow which occurred after administration of lead (described in 4.2.2.1). The biliary lead excretion increased during hours 4 and 5 after treatment, reaching an approximately 4 fold increment during the 5th hour over the level prior to lead infusion. Urinary lead excretion remained generally steady during the first 4 hours after treatment, and increased substantially during the 5th hour. At hour 5 after treatment, excretion in urine increased to a level approximately 18 times greater than the excretion level before lead administration. Figure 4.2.1 shows the interrelationship among changes in blood lead

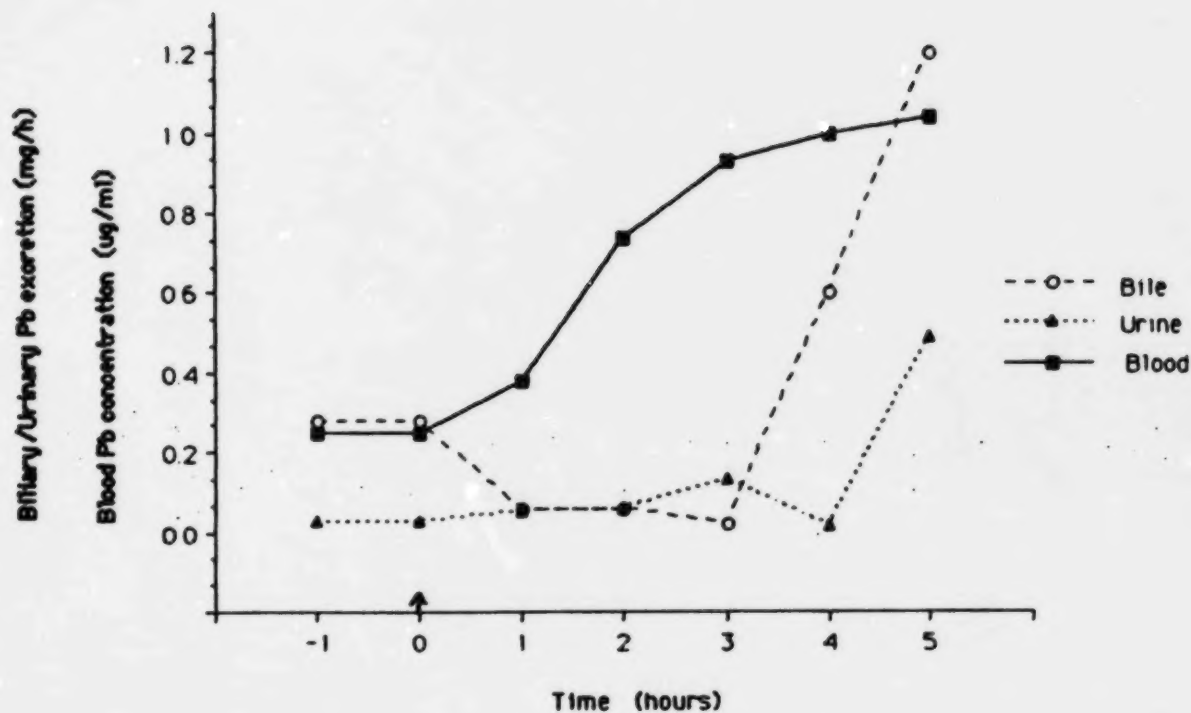


Figure 4.2.1 Blood lead concentration and biliary and urinary lead excretion prior to and after intraduodenal administration of lead acetate (10 mg Pb/kg).

Lead was administered at hour 0. The presented values are the average readings from 3 animals.

concentration and biliary and urinary lead excretion after intraduodenal administration of lead acetate.

4.2.2.2.2 The effect of thiamine, EDTA, and the combination of thiamine and EDTA on blood lead concentration.

A single injection with thiamine resulted in increased blood lead concentration. The increment in concentration was about 60% at the highest point, during the 3rd hour after treatment. The blood lead content returned to the level prior to thiamine injection at hour 4 and slightly increased during the 5th hour. Administration of EDTA brought about an increase in blood lead content during the first hour post treatment, followed by a decrease during the next 3 hours. At hour 4 the blood lead concentration fell approximately 25%. The blood lead began to increase during the 5th hour after treatment. Simultaneous administration of EDTA and thiamine augmented blood lead concentration at hour 1, then during the next 4 hours substantial reduction of lead in the blood occurred. Five hours after treatment the blood lead concentration decreased about 44%. Figure 4.2.2 presents changes in blood lead content after treatment with thiamine, EDTA, and EDTA plus thiamine.

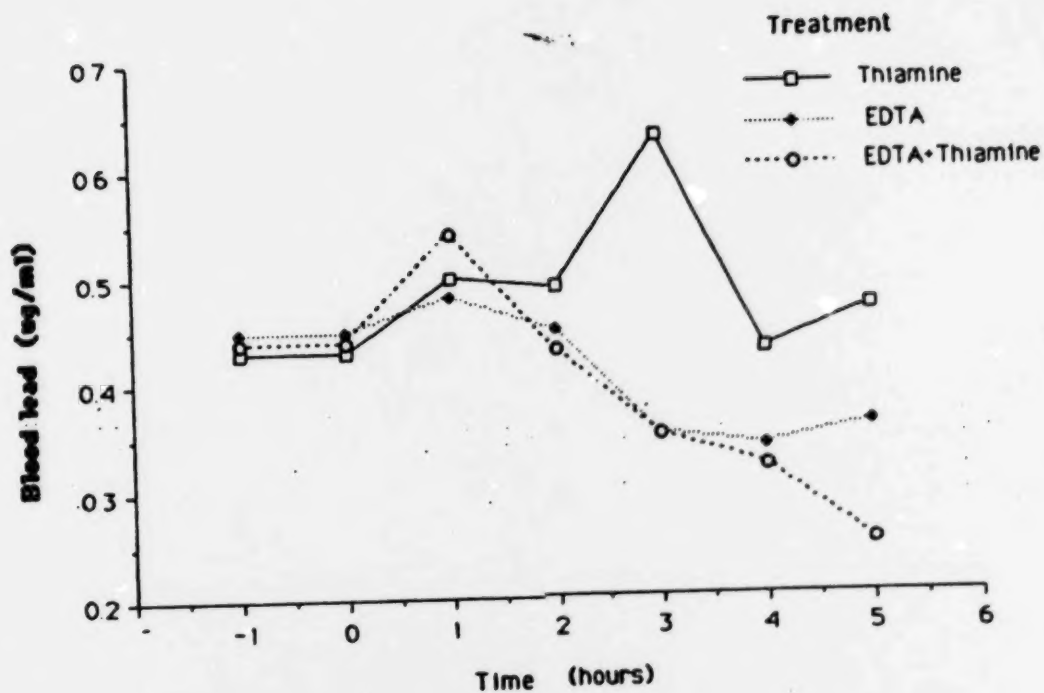


Figure 4.2.2 Blood lead concentration prior to and after treatment with thiamine, EDTA, and a combination of thiamine and EDTA in cows.

Treatment was administered at hour 0. The presented values are the average readings from 3 animals.

4.2.2.2.3 The effect of thiamine, EDTA, and the combination of EDTA and thiamine on biliary and urinary excretion of thiamine, copper, zinc, iron, and manganese.

4.2.2.2.3.1 Biliary excretion

Subcutaneously injected thiamine is excreted in bile, but co-treatment with EDTA did not affect normal physiological thiamine excretion in bile. Treatment with thiamine alone significantly increased biliary lead evacuation. Administration of both EDTA alone and in combination with thiamine resulted in lowering of the biliary lead excretion rate, as compared to basal level of excretion. Excretion in bile of copper, zinc, iron, and manganese was generally not affected by treatment with thiamine, EDTA, or EDTA and thiamine. The summary of results for biliary excretion is shown in Table 4.2.2.

4.2.2.2.3.2 Urinary excretion

Injected thiamine was rapidly eliminated in urine. The elimination rate was enhanced by simultaneous injection of EDTA. Treatment with EDTA alone increased excretion of thiamine as compared to basal level. Administration of thiamine enhanced urinary elimination of lead approximately 3 fold, whereas the effect of EDTA alone was about 35 times stronger than the effect of thiamine alone. The combination

TABLE 4.2.2 Biliary excretion of B_1 , Pb, Cu, Fe, and Mn: Basal level and effect after administration of thiamine, EDTA, and EDTA + thiamine in cattle pretreated with lead acetate.

Variable	Treatment				ANOVA P<
	Basal	Thiamine	EDTA	EDTA + Thiamine	
	mg/5 hours ^a				
(n)	3	3	3	3	
B_1	0.017 ^b ±0.006	11.64 ^d ±2.49	0.018 ^b ±0.002	6.4 ^c ±1.27	0.0009
Pb	1.94 ^c ±0.48	3.04 ^d ±0.25	1.05 ^{bc} ±0.4	0.52 ^b ±0.04	0.0035
Cu	1.1±0.3	1.17±0.23	0.94±0.1	0.69±0.08	0.3822
Zn	4.78±0.83	4.38±0.7	4.22±0.26	3.75±0.54	0.3837
Fe	8.70±1.82	8.13±0.39	8.06±0.89	8.95±1.41	0.9430
Mn	1.95±0.94	0.85±0.09	0.61±0.18	0.49±0.04	0.2037

n Refers to number of animals sampled per treatment.

^a Results are expressed as mean ± SE.

bcd Values with different superscripts within the row are significantly different (p<0.05).

treatment of EDTA and thiamine on average did not significantly affect urinary lead excretion as compared to EDTA effect alone. However, there were differences in response to treatment among individual animals. Treatment with EDTA significantly increased urinary zinc excretion. This effect of EDTA on zinc was enhanced by co-treatment with thiamine, but thiamine treatment alone did not affect urinary zinc excretion. Neither EDTA nor thiamine affected urinary iron. However, the combination treatment significantly increased excretion of iron in urine. Thiamine alone did not affect urinary manganese, but the combination treatment significantly increased excretion of manganese in urine in comparison to thiamine alone. Also, EDTA increased urinary excretion of manganese. Urinary copper excretion was not affected by any of the treatments (Table 4.2.3).

4.2.2.2.3.3 Total excretion

The effect of thiamine treatment on total excretion of lead was not significant, although the treatment increased evacuation of lead from the body in comparison to basal level. EDTA significantly increased lead excretion. EDTA treatment was approximately 10 times more effective than thiamine treatment in elimination of lead from the body and 18 times more effective than natural clearance. Cotreatment with thiamine resulted in decreased total lead excretion as compared to the EDTA effect, but the change was not

TABLE 4.2.3 Urinary excretion of B_1 , Pb, Cu, Fe, and Mn: Basal level and effect after administration of thiamine, EDTA, and EDTA + thiamine in cattle pretreated with lead acetate.

Variable	Treatment				ANOVA P<
	Basal	Thiamine	EDTA mg/5 hours ^a	EDTA + Thiamine	
(n)	3	3	3	3	
B_1	1.04 ^b +0.13	3193.26 ^c +966.63	3.38 ^b +0.40	3818.31 ^c +291.05	0.0007
Pb	0.41 ^b +0.17	1.19 ^b +0.36	42.13 ^c +15.81	39.44 ^c +7.13	0.0121
Cu	0.78+0.15	1.28+0.45	0.68+0.12	0.98+0.09	0.3942
Zn	3.45 ^b +0.49	3.55 ^b +1.04	45.31 ^c +16.26	54.99 ^c +4.95	0.0041
Fe	8.46 ^b +1.29	8.3 ^b +2.56	7.82 ^b +0.85	14.27 ^c +1.08	0.0399
Mn	0.41 ^b +0.1	0.59 ^{bc} +0.16	1.05 ^{cd} +0.31	1.34 ^d +0.09	0.0298

n Refers to number of animals sampled per treatment.

^a Results are expressed as mean $\pm$ SE.

bcd Values with different superscripts within the row are significantly different ($p < 0.05$).

significantly different. Both EDTA alone and in combination with thiamine significantly increased total zinc excretion, but thiamine alone did not have any effect.

Excretion of copper, iron, and manganese was not affected by any of the treatments. About 50% of injected thiamine was excreted within 5 hours after injection. Approximately 3 times more thiamine was excreted after treatment with EDTA in comparison to basal level (Figure 4.2.3).

4.2.2.2.3.4 Total excretion of lead with proportional relation to biliary and urinary excretion in cattle: Basal level and after treatment with thiamine and/or EDTA.

Treatment with thiamine increased biliary and urinary lead excretion approximately 57% and 190% respectively. The total lead elimination was increased by 80% after treatment with thiamine. EDTA increased lead excretion via urine 10000% but decreased biliary excretion about 45%. Total lead excretion after EDTA administration was increased 1838%. Combination treatment increased urinary lead excretion but less effectively than EDTA alone. Biliary lead excretion was lowered approximately 273% by this treatment (Figure 4.2.4).

4.2.2.3 Discussion

Intraduodenal administration of lead acetate resulted in

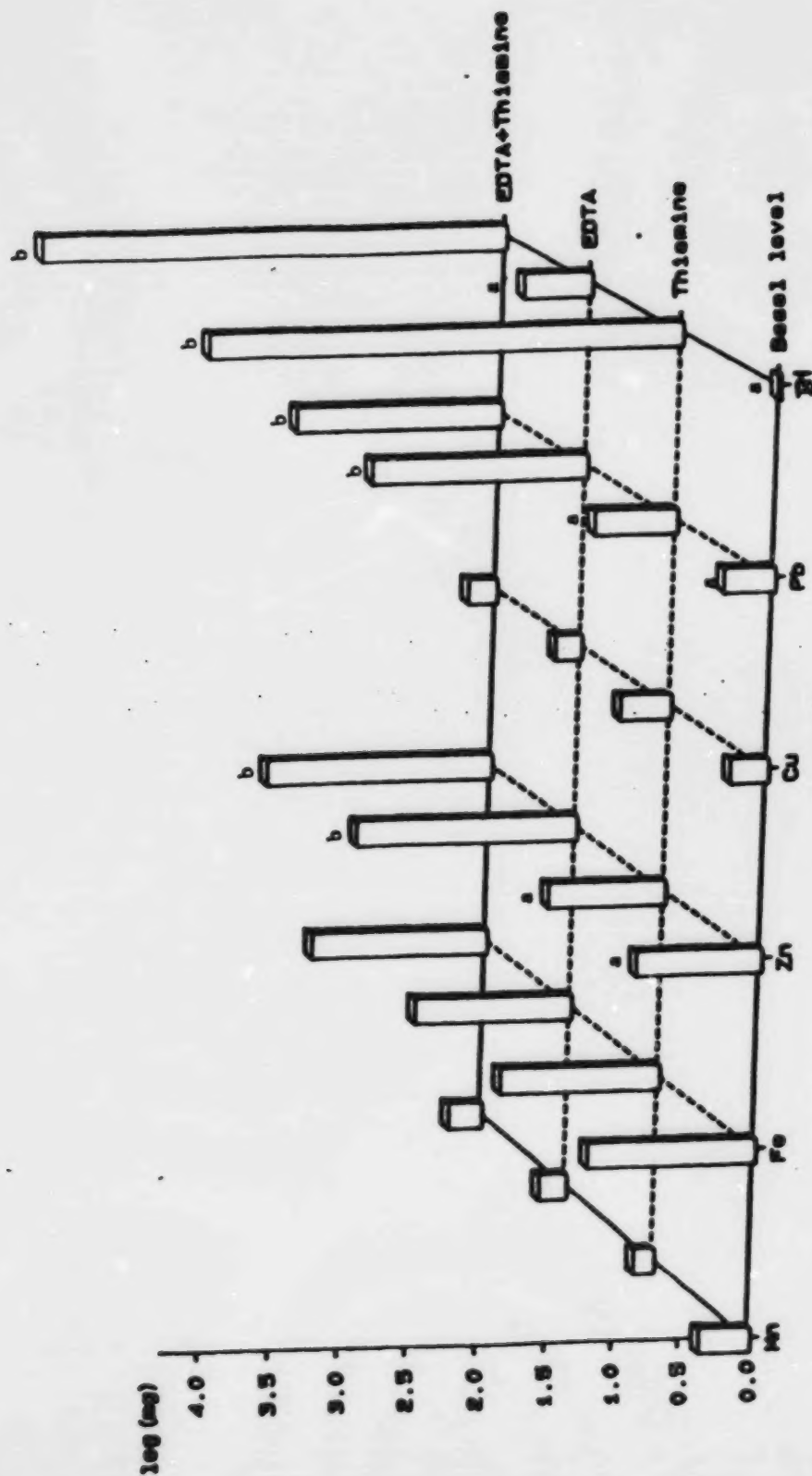


FIGURE 4.2.3 Total cumulative excretion (5 hours) of thiamine (B1), Lead (Pb) copper (Cu), zinc (Zn), iron (Fe), and manganese (Mn) after administration of thiamine, EDTA, and EDTA + thiamine in comparison with basal level of excretion in cattle.

* a, b values with different letters on "z" axis are significantly different (p 0.05)

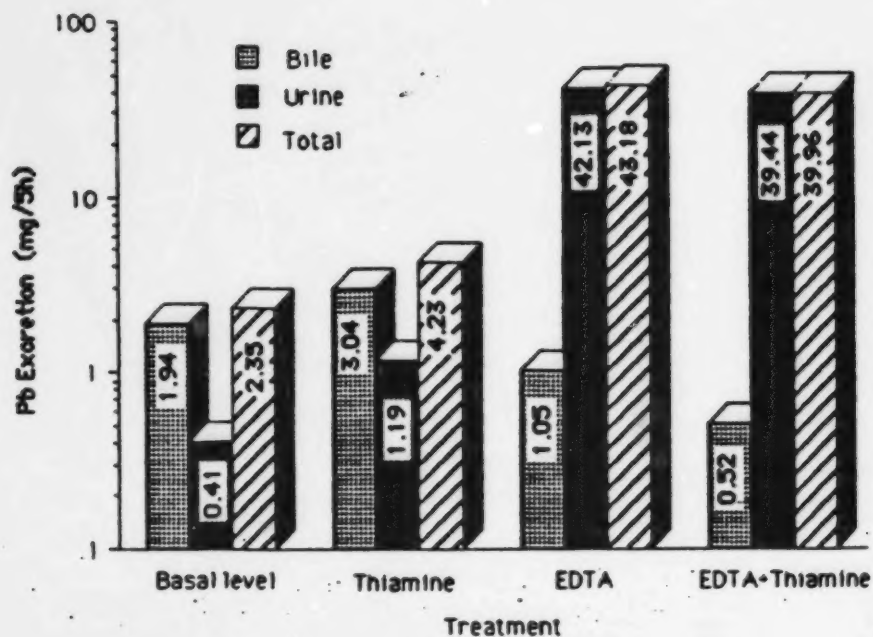


Figure 4.2.4 Total excretion of lead with proportional relation to biliary and urinary excretion in cattle: Basal level and after treatment with thiamine and/or EDTA.

The graph is presented on logarithmic scale. The figures printed on bars represent untransformed average values of 3 animals.

a drastic increase in blood lead concentration followed by an increase in biliary and urinary lead concentration. Ito et.al (1987) hypothesized that thiamine may enhance the active transport mechanism of biliary lead secretion. This assumption was generally confirmed in this study. A single dose of thiamine administered subcutaneously increased biliary lead excretion in cattle. Evacuation of lead via bile appears to be an effective route of eliminating lead from the organism. Only 0.3% lead excreted in bile was shown to undergo enterohepatic circulation in rats (Klassen and Shoeman 1974). Flora et. al (1986) reported that thiamine increased urinary lead excretion and enhanced the effect of EDTA. Similar findings were now obtained in this experiment with regard to the thiamine alone effect on urinary lead, but only in one case out of three did thiamine enhance the effect of EDTA on urinary lead excretion. In the remaining two animals excretion was decreased by the combination of thiamine and EDTA treatment as compared to EDTA alone.

Urinary excretion of copper, zinc, iron, and manganese was reported to be increased by chelation therapy with EDTA (Victory et al 1986, Cantilena and Klaassen 1982). In the present study, increased urinary excretion of zinc and manganese was observed after administration of EDTA, and co-treatment with thiamine further enhanced excretion of these metals. Combination treatment also resulted in increased excretion of iron in urine. None of the treatments affected

urinary excretion of copper.

Biliary excretion of copper, zinc, iron, and manganese was not affected by any of the treatments.

The findings with respect to the effect of thiamine on blood lead concentration obtained from different experiments are not consistent. Bratton et al (1981), Flora et al (1986) and Kim (1988) reported that treatment with thiamine decreased blood lead level, but Kachru et al (1987) showed that thiamine injection increased blood lead content. In the present study, the blood lead level was found to be elevated shortly after thiamine injection. These discrepancies may be due to differences in species used and in experimental design. EDTA decreased blood lead concentration, and thiamine considerably enhanced the effect of EDTA.

4.3 Lead Excretion after challenge with Thiamine and/or EDTA in lead loaded sheep: The effects on metabolism of copper, zinc, iron, and manganese.

4.3.1 Materials and Methods

4.3.1.1 Animals and diets

Five cross breed female sheep approximately 3 months of age at the commencement of the experiment were housed in individual pens. The sheep had free access to tap water and were offered the experimental diet at the rate of 1 kg/day per animal. The diet was formulated to meet the minimum nutritional requirement for sheep (NRC-NAS 1984) and contained 596 ppm of lead. The composition and mineral analysis of the diet is shown in Appendix B, Table 1. After 30 days of conditioning on the diet, all animals were surgically modified to allow collecting of bile samples and measuring bile flow without interfering with digestive processes.

4.3.1.1.1 Surgical procedure

Ligation of bile duct and cannulation of gall bladder and duodenum were performed as described by Caple and Heath (1972) with minor modifications.

Briefly; The animals were anesthetized and placed in left lateral recumbency. After clipping and aseptically

preparing the surgical site, the area was draped with sterile drapes. Aseptic technique was followed through the remainder of the procedure. A 10 inch, right para costal incision was made through the skin and abdominal musculature. The common bile duct in the proximity of the pancreas was identified, isolated from the surrounding tissue, creating as little trauma to the pancreas as possible, and double ligated using #2-0 polyglycolic acid suture. The duct was not transected in this procedure. The distal free end of the gall bladder was further separated from its attachment to the visceral surface of the liver taking care during this dissection not to invade the liver tissue itself. An 18gg needle on a 30ml syringe was used to aspirate the contents of the gall bladder at this time. Using #2-0 polyglycolic acid suture, a purse string suture pattern was pre-placed in the distal end of the gall bladder and a small stab incision made within the perimeter of this suture pattern. The flange of the collecting cannula was inserted through this stab incision and the purse string suture secured. The plastic washer and marlex mesh was placed over the cannula and the cannula exited through a small stab incision in the body wall situated cranial to the original skin incision. A second washer was placed over the cannula and brought to rest against the skin. Care was taken to ensure the gall bladder was snugly sandwiched between the inner flange of the collecting cannula and the body wall to allow maximum contact

and hence optimal fibrosis between these structures in order to create a leak proof adhesion. During placement of the bile cannula, care was taken to minimize spillage of bile and contamination of the abdomen. A portion of the duodenum, distal to the point of entry of the pancreatic duct was used for the placement of the re-entrant cannula. A purse string suture using #2-0 polyglycolic acid suture was pre-placed on the antimesenteric side of the duodenum and an incision made to allow insertion of the T portion of the re-entrant cannula. The suture was secured and this cannula was exited through the body wall through a stab incision caudal to the skin incision and ventral to the exit point of the bile cannula. Care was taken not to make either exiting stab incision too large as previous experience had shown this lead to catheters being accidentally pulled out. An external washer was again placed on this cannula and again snug contact between the bowel and body was established. The abdominal musculature was closed in a single layer using a continuous d#0 polyglycolic suture and the skin closed with a #0 supramid in a continuous interlocking pattern. The cannulae were secured and stabilized by wrapping them with adhesive tape close to their point of emergence through the skin again ensuring an adequate contact between either the gall bladder or duodenum and the body wall. The two cannulae were connected externally with a plastic tubing containing a one way valve system.

4.3.1.2 Experimental design

The experiment was carried out in the form of six consecutive treatments, each followed by a sampling session (Table 4.3.1). Prior to each treatment every sheep received 20 mg/kg BW per day of lead as aqueous solution of lead acetate (BDH Chemicals) 250 mg Pb/100 ml. Lead acetate solution was administered through intraduodenal cannulae in equal daily doses at 9:00 am, 12:00 pm, 3:00 pm, and 6:00 pm for 2 days. The treatments were administered identically to all sheep throughout the experiment. However, one sheep, (#304), served to test the safety of the planned dose of thiamine and/or EDTA. This animal received each treatment immediately after the last intraduodenal infusion of lead and was not sampled, but observed for toxic effects of the agent used for treatment. The remaining sheep received treatments about 16 hours after the last infusion of lead, and each treatment was followed by a sampling session (Table 4.3.1).

4.3.1.3 Sampling schedule

Samples of blood, bile, and urine were collected for one hour prior to each treatment. Following the treatments, a general sampling schedule was adopted as follows:

Bile: For the first 24 hours after treatment samples were obtained every half hour. Then at hour 27, 30, 33, 45, 51, 57, and 69, half-hour samples were taken.

Table 4.3.1 Schematic summary of experimental design.

Trial	(n)	Treatment	Sampling (hours)
A	4	two doses of Pb (10 mg/kg at hour 0) (15 mg/kg at hour 3)	6
B	4	Thiamine 37.5 mg/kg BW	30
C	4	Thiamine 75 mg/kg BW	69
D	3	Thiamine 150 mg/kg BW	69
E	3	EDTA 110 mg/kg BW	69
F	3	Thiamine 75 mg/kg BW + EDTA 110 mg/kg BW	117 ^a

n refers to number of animals sampled per treatment.

a samples were collected for 117h, however since Pb excretion was not increased after 69h all statistical analysis are based on 69h sampling period.

Urine: For the first 6 hours after treatment samples were obtained every one hour. Then from hour 6 to hour 33, 3-hour samples; from hour 45 to hour 57, 6-hour samples; and finally at hour 69, half-hour samples were collected.

Blood: For the first 6 hours after treatment samples were obtained every one hour; then from hour 6 to hour 33, every 3 hours; and thereafter at hour 45, 51, 57, and 69.

4.3.1.4 Sample collection

Samples of bile, urine and blood were collected as described in section 4.2.1.4 (for urine collection, Foley catheter 12 Fr/10 cc was used).

4.3.1.5 Measurements

4.3.1.5.1 Clinical features

Clinical appearance and vital neurological signs were monitored twice a day throughout the experiment.

4.3.1.5.2 Analytical measurements

Analysis of thiamine was performed as described in section 3. Analysis of lead, copper, zinc, iron, and manganese was performed as described in section 4.2.1.5.2.

4.3.1.6 Analysis of data

Statistical analysis was performed as described in section 4.2.1.6.

4.3.1.7 Basal level of excretion

Basal level of excretion was established according to the same assumptions as described in section 4.2.1.7.

4.3.2 Results

4.3.2.1 Clinical Features

Neurological signs were not seen throughout the experiment. Decreased feed intake and transient mild depressions were observed on the second day of lead infusion. One sheep (#349) developed extensive inflammatory response around the jugular vein on both sides and was eliminated from experiments E and F.

4.3.2.2 Analytical Data

4.3.2.2.1 Changes in blood content and biliary and urinary excretion of lead after intraduodenal administration of lead acetate in lead loaded sheep.

In sheep chronically exposed to lead the intraduodenal challenge with lead acetate resulted only in a mild increase

of blood lead concentration. In response to a second challenge, blood lead increase was slightly more pronounced. Both biliary and urinary lead excretion increased after lead administration, but the responses after the second infusion were of higher magnitude (Figure 4.3.1).

4.3.2.2.2 The interrelationship of biliary and urinary excretion of lead and thiamine.

The increase in biliary and urinary lead excretion was accompanied by a simultaneous increase in biliary and urinary thiamine excretion. The urinary thiamine was elevated 1 hour after first lead infusion and then returned to basal level. The same pattern was followed after a second lead infusion. The biliary thiamine excretion was rising steadily during the interval between first and second lead administration, but second lead infusion resulted in a substantial increase in the relative excretion rate of thiamine as compared to the effect after the first infusion (Figure 4.3.2). There was no change in blood thiamine content.

4.3.2.2.3 Thiamine blood concentration and biliary and urinary excretion after subcutaneous administration of thiamine HCl.

No toxic effects of thiamine were noted after injection of thiamine at any of the tested doses. The general pattern was similar for each dose and specimen, however, magnitude

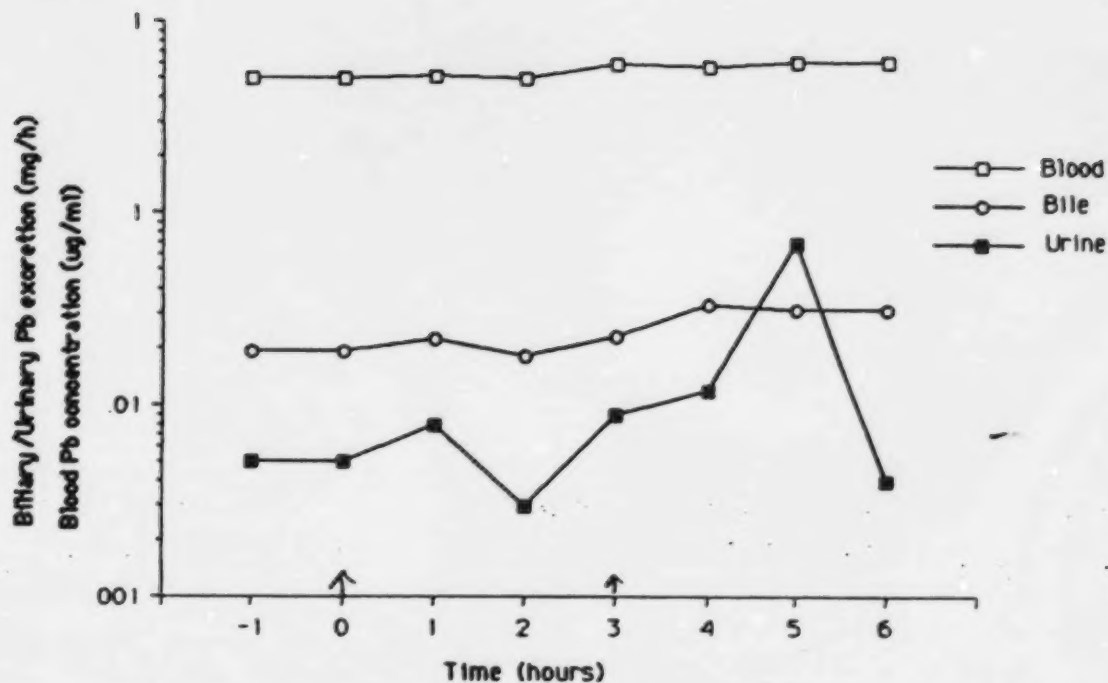


Figure 4.3.1: Blood lead concentration and biliary and urinary excretion prior to and after intra-duodenal challenge with lead acetate.

Lead was administered at hour 0 (10 mg Pb/kg) and at hour 3 (15 mg Pb/kg). The presented values are the average readings from 4 animals.

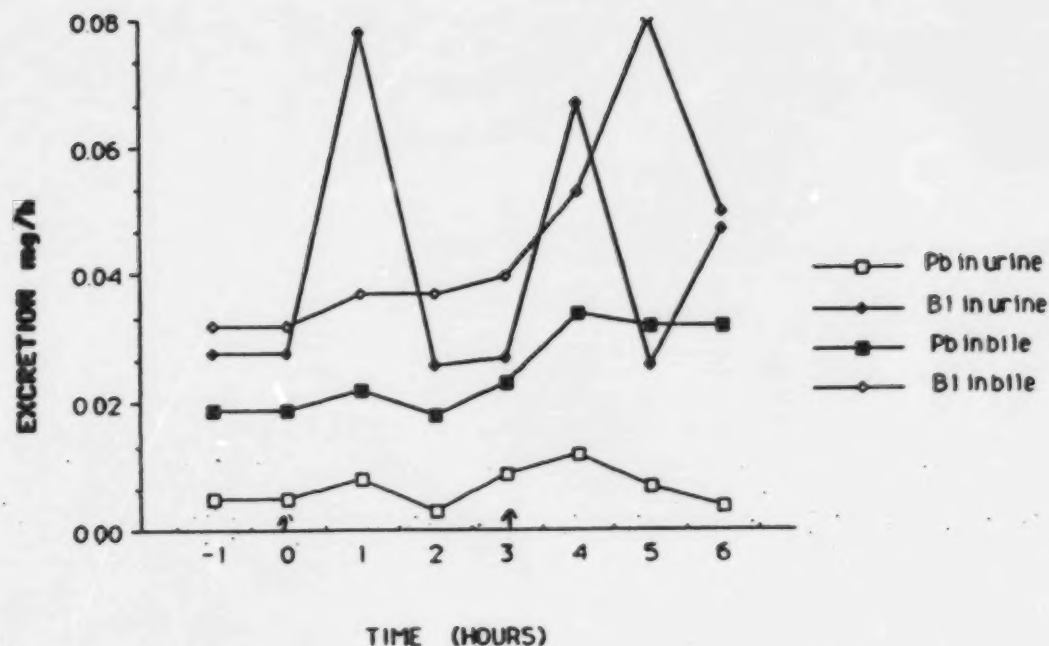


Figure 4.3.2 Interrelationship between thiamine and lead excreted in bile and urine. The effect of intraduodenal administration of lead acetate.

Lead was administered at hour 0 (10 mg Pb/kg) and at hour 3 (15 mg Pb/kg). The presented values are the average readings from 4 animals.

increased respectively to dose. Certain differences were noted among individuals with respect to temporal expression of excretion peak as well as magnitude. Blood thiamine concentration and urinary excretion reached their peaks approximately 1 - 2 hours after injection. The highest excretion in bile was between hours 3 - 4. The 75 mg/kg dose resulted in approximately 1000 fold increment in blood thiamine level. The excretion in bile and urine increased approximately 10^4 fold at peak of excretion.

The pattern for all three specimens can generally be fit into a biphasic model with clearly distinguishable aspects of faster and slower rates of changes (Figure 4.3.3).

4.3.2.2.4 The effect of thiamine dose on excretion of lead.

The total output of lead was not affected by the dose of thiamine injected. The response to thiamine varied among individuals with respect to the distribution of lead excreted in the bile or urine and was also not affected by the dose (Figure 4.3.4).

4.3.2.2.5 The effect of thiamine, EDTA, and the combination of EDTA and thiamine on biliary and urinary evacuation of lead, copper, zinc, iron, and manganese in lead loaded sheep.

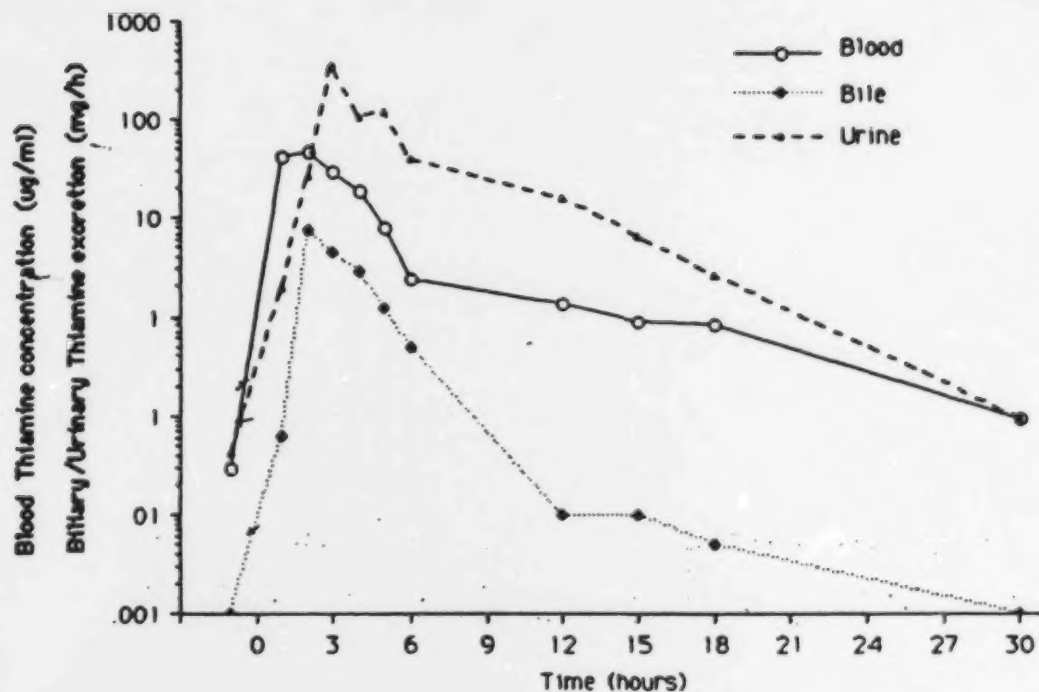


Figure 4.3.3 Changes in blood thiamine concentration and biliary and urinary excretion profiles during 69 hour sampling period after subcutaneous administration of 75 mg/kg of thiamine HCl.

The presented values are the actual readings from one sheep (#366), since variability among individual animals would distort the profile if readings were averaged (see section 4.3.2.2.3).

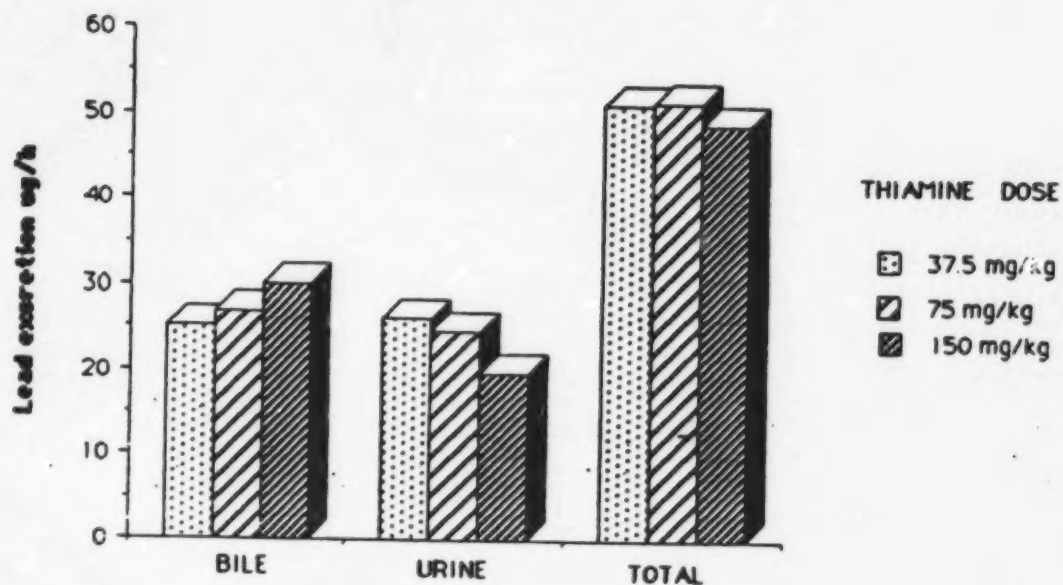


Figure 4.3.4 Effect of thiamine dose on biliary and urinary excretion of lead in sheep.

The presented values are the average readings from 2 animals.

4.3.2.2.5.1 Biliary excretion

None of the applied treatments brought about significant changes in biliary lead excretion. However, treatment with thiamine alone and in combination with EDTA resulted in a slightly higher excretion rate than basal level in some sheep. (For raw data see Table 3, App. B.) Treatment with thiamine alone and EDTA alone significantly reduced biliary excretion of iron as compared to the combination treatment, but there was no significant change between basal excretion and any of the treatments.

Biliary excretion of copper, zinc, and manganese was not affected by treatments. Both treatments involving injection of thiamine resulted in a significant increase of thiamine in bile (Table 4.3.2).

4.3.2.2.5.2 Urinary excretion

Thiamine increased urinary lead excretion in all sheep (for raw data see Table 3. App. B), although the overall change was statistically not significant. The administration of EDTA resulted in significant increase in elimination of lead via urine. Thiamine enhanced the effect of EDTA, however not significantly. Urinary excretion of copper, zinc, iron, and manganese was not affected by treatment in terms of statistical analysis, but substantial variability was noted among individual animals with respect to zinc, iron, and manganese. (For raw data see Table 3. App. B.)

TABLE 4.3.2 Biliary excretion of B_1 , Pb, Cu, Fe, and Mn: Basal level and effect after administration of thiamine, EDTA, and EDTA + thiamine in lead loaded sheep.

Variable	Treatment				ANOVA P<
	Basal	Thiamine	EDTA	EDTA + Thiamine	
	mg/69 hours ^a				
(n)	4	4	3	3	
B_1	0.004 ^b ±0.01	10.93 ^c ±2.86	0.1 ^b ±0.04	17.76 ^c ±5.68	0.0041
Pb	1.03±0.18	1.31±0.37	0.97±0.26	2.37±1.08	0.2894
Cu	1.03±0.42	1.03±0.31	0.46±0.12	1.68±1.09	0.5711
Zn	2.45±0.15	2.69±0.62	2.14±0.51	4.26±2.39	0.5927
Fe	6.09 ^{bc} ±0.21	2.28 ^b ±0.26	2.29 ^b ±0.28	12.1 ^c ±5.91	0.0698
Mn	0.70±0.17	1.08±0.17	1.16±0.6	2.20±0.88	0.2495

n Refers to number of animals sampled per treatment.

^a Results are expressed as mean ± SE.

^{bc} Values with different superscripts within the row are significantly different ($p < 0.05$).

Thiamine excretion in urine was significantly different for treatments which received thiamine injection as compared to those which did not. The summary of results are presented in Table 4.3.3.

4.3.2.2.5.3 Total excretion

Sheep treated with thiamine excreted more lead as compared to basal level of excretion, however the change was not significant at level $p < 0.05$. EDTA treatment resulted in significantly enhanced elimination of lead from the body. The excretion was 3.5 times greater than after treatment with thiamine and approximately 6 times greater than basal level. The EDTA and thiamine combined treatment was the most efficient one. Thiamine enhanced the effect of EDTA on total excretion by 58% and the change was statistically significant ($p < 0.05$). Total excretion of zinc was significantly increased by the combination treatment in comparison to treatment with thiamine alone. Changes in excretion of zinc were insignificant between basal level and any of the treatments, as well as between EDTA and EDTA plus thiamine treatments. There was no effect of thiamine alone and EDTA alone treatments on iron excretion, but the combination treatment brought about a significant increase of total iron excreted from the body. Excretion of copper and manganese was not affected by any of the treatments, but manganese excretion varied considerably among individuals.

TABLE 4.3.3 Urinary excretion of B_1 , Pb, Cu, Fe, and Mn: Basal level and effect after administration of thiamine, EDTA, and EDTA + thiamine in lead loaded sheep.

Variable	Treatment				ANOVA P<
	Basal	Thiamine	EDTA	EDTA + Thiamine	
	mg/69 hours ^a				
(n)	4	4	3	3	
B_1	2.34 ^b ±0.96	1225.18 ^c ±248.25	4.42 ^b ±0.45	1123.30 ^c ±161.68	0.0002
Pb	0.37 ^b ±0.12	1.09 ^b ±0.10	7.35 ^c ±0.28	10.73 ^c ±1.05	0.0001
Cu	0.55±0.28	0.47±0.05	0.98±0.39	0.94±0.33	0.4620
Zn	4.97±0.85	3.00±0.64	9.15±3.76	9.17±2.53	0.1264
Fe	3.36±1.10	3.76±2.29	2.56±0.25	14.89±9.79	0.2432
Mn	0.26±0.07	90.73±61.56	162.90±103.35	117.57±111.1	0.4696

n Refers to number of animals sampled per treatment.

^a Results are expressed as mean ± SE.

^{bc} Values with different superscripts within the row are significantly different ($p < 0.05$).

Approximately 75% of the subcutaneously administered thiamine was excreted after 69 hours. The excretion of thiamine was higher after treatment with EDTA than basal level, but the difference was not significant (Figure 4.3.5).

4.3.2.2.5.4 Comparison of biliary and urinary lead excretion and their proportions to total lead excretion.

About three times more lead was eliminated through bile than in urine at basal level of excretion. Treatment with thiamine increased biliary lead approximately 27% and urinary lead approximately 295%. Total lead evacuation was increased by about 73% upon treatment with thiamine. EDTA considerably enhanced urinary, but slightly decreased biliary lead excretion. The increment in urinary lead was approximately 2000%. Total lead excretion was increased by EDTA about 600%. The combination of EDTA and thiamine increased biliary lead excretion 230% and urinary lead excretion 2900%. Total excretion of lead was enhanced by the combination treatment approximately 942% (Figure 4.3.6).

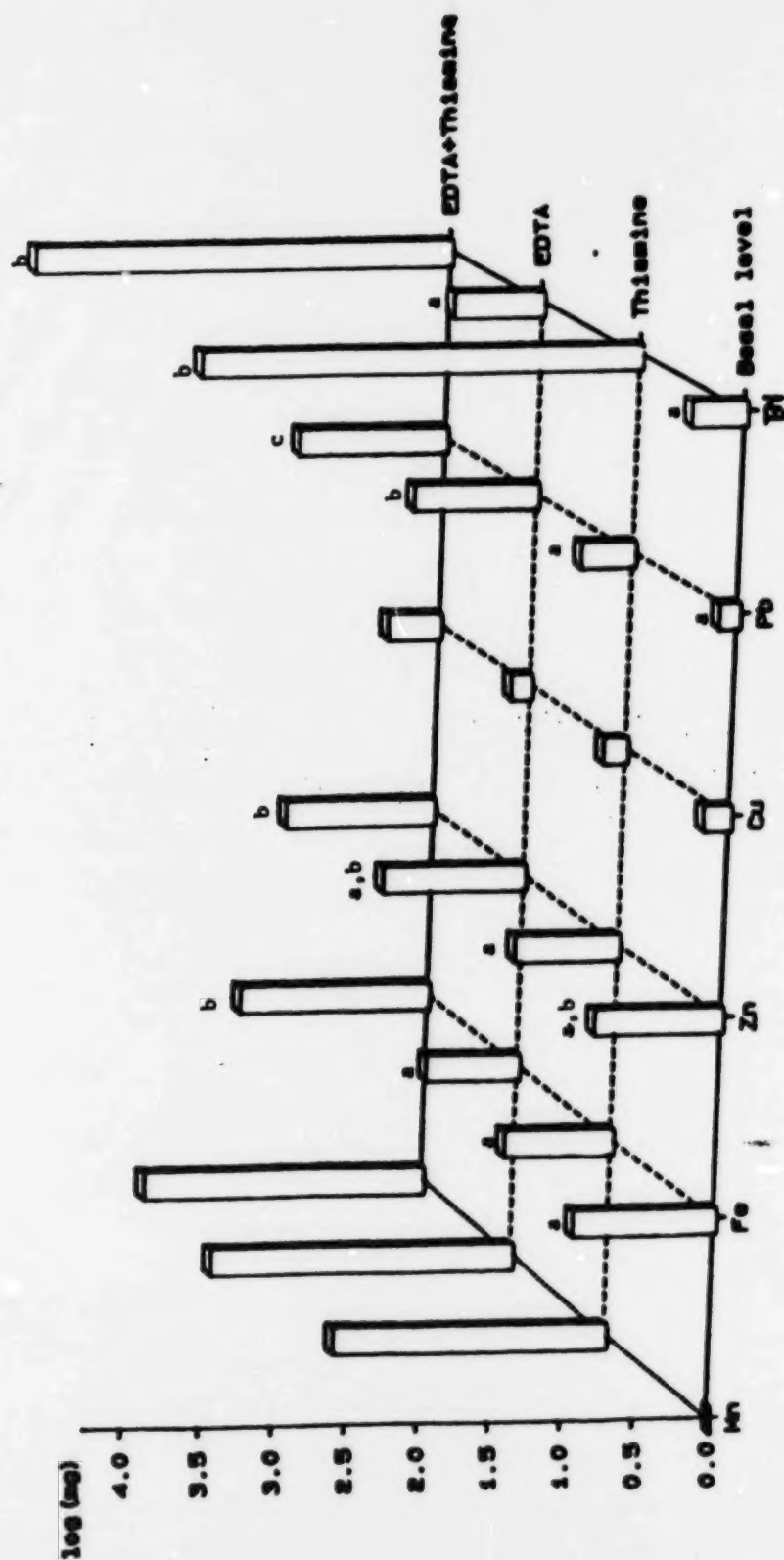


FIGURE 4.3.5 Total cumulative excretion (69 hours) of thiamine (Bl), lead (Pb), copper (Cu), zinc (Zn), iron (Fe), and manganese (Mn) after administration of thiamine, EDTA, and EDTA + thiamine in comparison with basal level of excretion in lead loaded sheep.

* a,b,c values with different letters on "z" axis are significantly different (p 0.05)

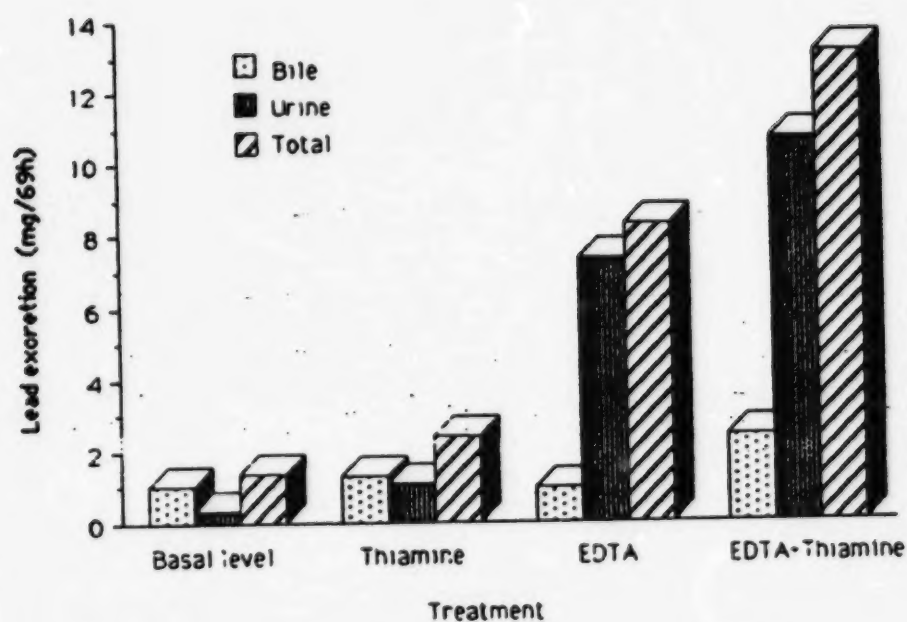


Figure 4.3.6 Comparison of biliary and urinary lead excretion and their proportions to total lead excretion.

4.3.2.2.6 Changes in blood lead concentration and biliary and urinary excretion during the 69 hour sampling period in response to treatment with thiamine, EDTA, and a combination of EDTA and thiamine: A selected case to study the model of metabolic interrelations.

4.3.2.2.6.1 Blood lead content

Administration of thiamine resulted in an increase in blood lead concentration. Blood lead decreased after treatment with EDTA as well as after the combination treatment although the combination treatment appeared to be more effective in lowering blood lead (Figure 4.3.7).

4.3.2.2.6.2 Biliary lead excretion

Biliary lead evacuation was enhanced by thiamine, but depressed by EDTA treatment. The combination of EDTA and thiamine also had a depressive effect on biliary lead excretion, although it was more pronounced in comparison with the EDTA alone effect (Figure 4.3.8).

4.3.2.2.6.3 Urinary lead excretion

Thiamine treatment increased elimination of lead via urine as compared to the level before thiamine injection. Treatment with EDTA alone resulted in drastically elevated urinary lead. Co-treatment with thiamine enhanced the effect

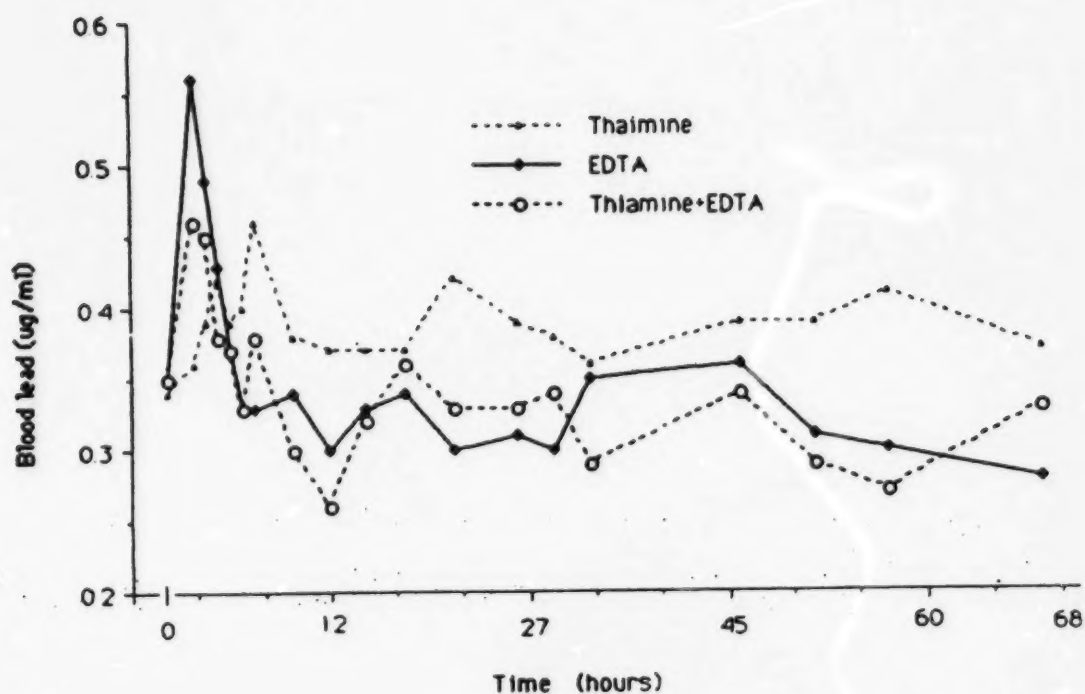


Figure 4.3.7 Comparison of blood lead concentration profiles during 69 hours after treatment with thiamine, EDTA, and combination of thiamine and EDTA.

The treatment was administered at hour 0. The presented values are the actual readings from sheep #366.

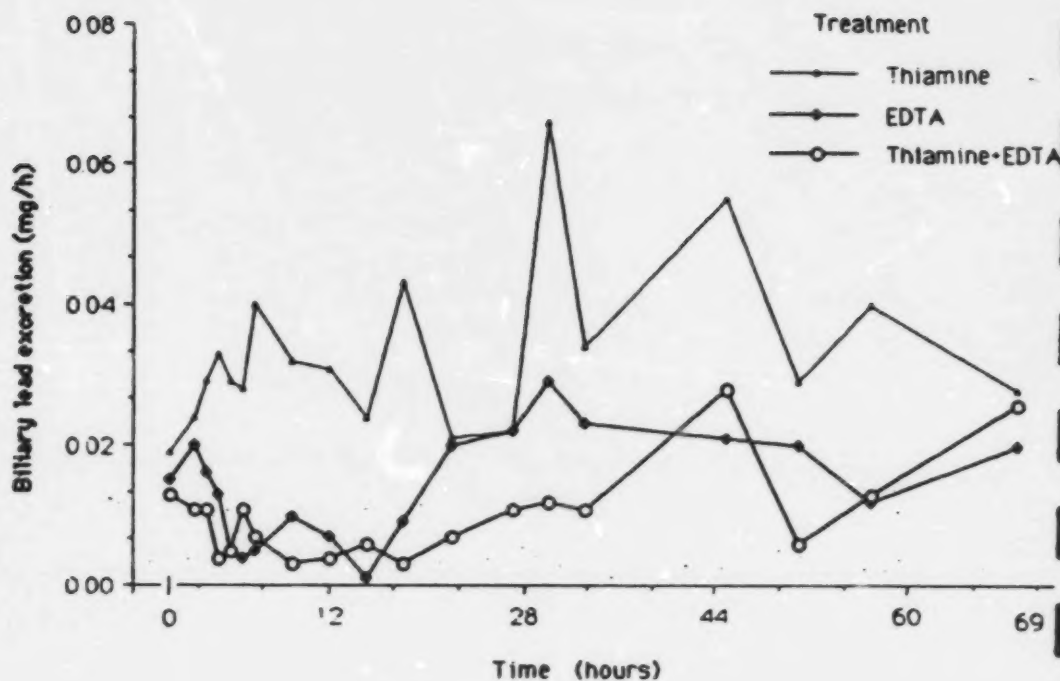


Figure 4.3.8 Biliary lead excretion profiles during 69 hour sampling period in response to treatment with thiamine, EDTA, and combination of thiamine and EDTA.

The treatment was administered at hour 0. The presented values are the actual readings from sheep #366.

of EDTA (Fig. 4.3.9).

4.3.2.2.6.4 The contrast in changes of bile:blood concentration ratio for lead and copper after treatment with thiamine.

The administration of thiamine did not affect the relationship between bile and blood with respect to copper, zinc, and manganese. On the other hand, the bile:blood concentration ratio for lead was considerably increased after treatment with thiamine. The pattern of the curve indicates a saturable system of lead elimination via bile upon treatment with thiamine. Since the lead analysis were performed on whole blood, the presented values in Figure 4.3.10 do not reflect a true relationship between the mobilizable pool of lead in the blood (plasma lead) and excreted lead in the bile. The plasma lead represents approximately 1% of the total blood lead, thus the ratios of bile:plasma lead would be about 100 times greater.

4.3.3 Discussion

Sheep chronically exposed to lead responded to intraduodenal administration of lead acetate by increased blood lead content and increased biliary and urinary excretion of lead. Generally speaking, the response was qualitatively similar to that observed in cattle (section 4.2), except of much lesser magnitude. Saturation of the

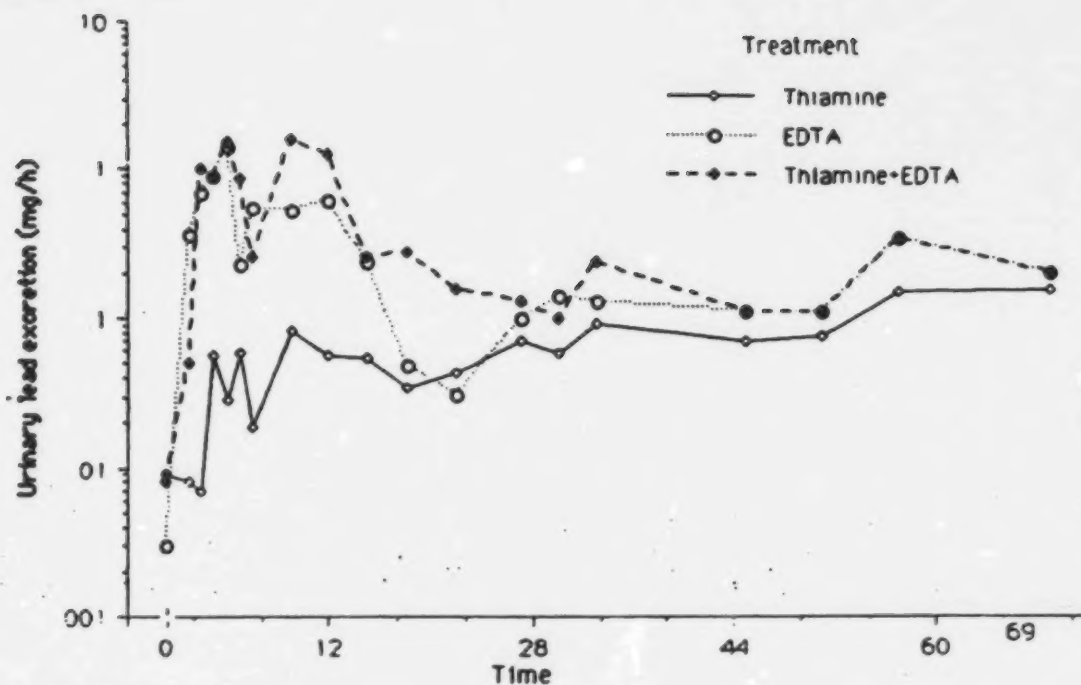


Figure 4.3.9 Urinary lead excretion profiles during 69 hour sampling period in response to treatment with thiamine, EDTA, and combination of thiamine and EDTA.

The treatment was administered at hour 0. The presented values are the actual readings from sheep #366.

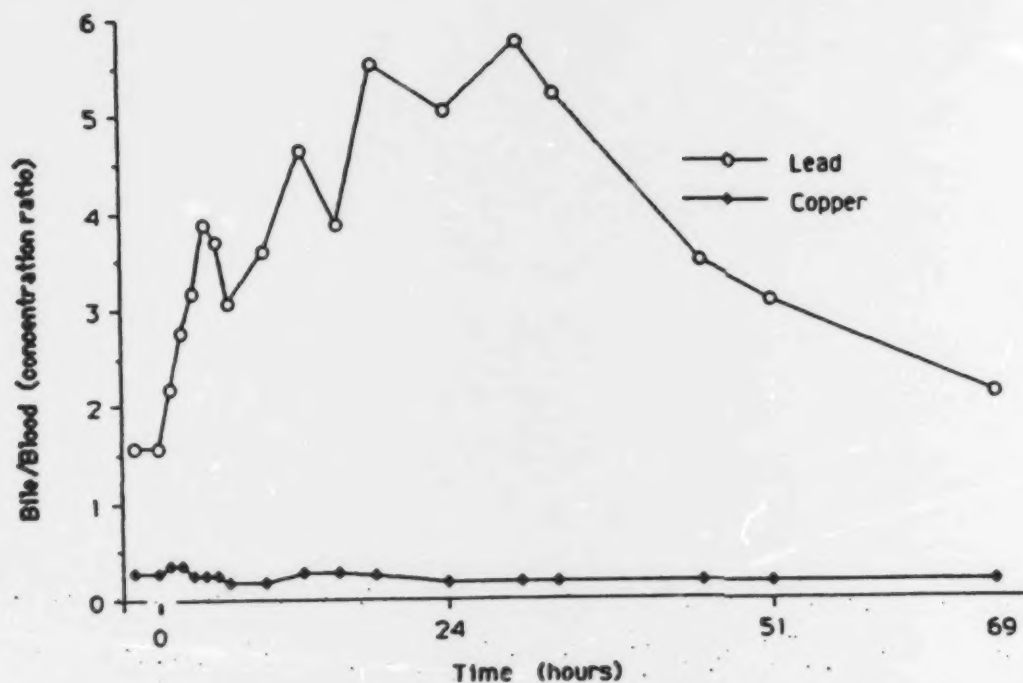


Figure 4.3.10 The contrast in changes of bile:blood concentration ratio for lead and copper after treatment with thiamine.

Bile:blood ratio profiles for zinc and manganese (not shown in Figure) were similar to those of copper. The treatment was administered at hour 0. The presented values are the actual readings from sheep #366.

system with lead could explain the relatively diminished response to intraduodenal challenge with lead in lead loaded sheep as compared to cattle exposed very little to lead. It appears that increase in excretion of lead both in bile and urine was accompanied by increased excretion of thiamine. The metabolic interaction of lead and thiamine are not well understood. Formation of lead-thiamine complexes can be hypothesized. The thiamine-metal (cadmium and copper) complexes have been obtained in vitro, and characterized by crystallography (Caira et al 1974, Cramer et al 1982). The significance of such complexes in biological systems is not known.

The total output of lead appears to be generally unchanged by the doses of thiamine tested in the present experiment. Considerable differences were observed between individual sheep with respect to distribution of amount of lead excreted in bile or urine. In response to increased dose, either the increase of lead excreted in bile was accompanied by a decrease of urinary excretion, or the relationship was reversed. But the total output was generally unchanged when comparing doses and/or individual sheep. Apparently, the ultimate capacity to eliminate lead from the body was exhausted by the injection of the lowest of the tested doses (ie: 37.5 mg/kg). In some sheep the higher dose of thiamine increased urine volume. Administered subcutaneously, thiamine has been reported to promote

urination in rabbits (Hecht and Weese 1937). This effect of thiamine could partially explain the changes in urinary or biliary output without changing the total output, but no regular pattern was observed. (For raw data see Table 4, App. B.)

The injection of thiamine, both alone and as a co-treatment with EDTA, greatly enhanced biliary and urinary excretion of lead in some sheep, but had only mild or no effect in others.

Findings from the present experiment indicated a substantial variability among individual sheep with respect to distribution of excreted lead in bile or urine. By analysing the combined urinary and biliary output as total body output, it was expected to decrease variability among individuals. Indeed, analysis revealed that thiamine enhanced the effect of EDTA, and the difference was statistically significant. The effects for both bile and urine separately were insignificant.

Upon analysing the model of metabolic effects after administration of thiamine and/or EDTA in lead loaded sheep, a few interesting observations surfaced. Thiamine alone increased blood lead, but administered as cotreatment with EDTA, it enhanced the effect of EDTA in lowering blood lead content. It appears that thiamine has remarkable capacity to mobilize intracellular lead, and if the system is not overwhelmed the chelating agent can effectively remove this

lead from the body. The present study provided evidence for an active transport system in bile with specificity for lead. Lead, but not copper, zinc, iron, or manganese was excreted against the concentration gradient in the bile. Klaassen and Shoeman (1974) put forward the hypothesis that lead in bile might be excreted by an active transport system. Thiamine did increase the excretion of lead in bile. Both rate of excretion and lead bile/blood ratio were elevated. Interestingly, EDTA alone and in combination with thiamine decreased biliary lead. These findings would indicate a strong dependence of biliary excretion of lead on the amount of unbound (excretable) lead in the blood.

4.4 Residue levels of lead, copper, zinc, iron and manganese in liver, kidney, heart, spleen, brain, and bone in sheep chronically exposed to lead in the diet: The effect of repeated injections of thiamine.

4.4.1 Materials and methods

Four sheep used for the experiment described in section 4.3 were utilized again for this experiment, after allowing an interval of 10 days during which they were fed the same experimental diet (Appendix B, Table 1). Housing, water, and feeding were identical to those described in section 4.3.1.1.

4.4.1.2 Experimental design

The sheep (Pb exposed) were randomly divided into 2 groups with 2 animals in each. One group (Pb exposed, B₁ treated) received injections of thiamine HCl (Rafter 8 Products, Calgary, AB) at a rate of 50 mg/kg BW subcutaneously at weekly intervals for 5 weeks. The other group (Pb exposed, untreated) did not receive any injections. At the end of the fifth week, all sheep were euthanised with barbiturate and autopsies were performed.

4.4.1.3 Sample collection

For mineral analysis samples of liver, kidney, heart, spleen, brain, and bone were taken and stored at -20° C. The tissues from two sheep (#202 and #238) fed the same basic

diet as Pb exposed sheep but without lead addition were used as normal control.

4.4.1.4 Measurements

4.4.1.4.1 Clinical features

Clinical features and vital neurological signs were monitored twice every day throughout the experiment.

4.4.1.4.2 Analytical measurements

For mineral analysis samples of soft tissues were freeze dried and bones were ashed. Digestion of samples and analytical procedure was performed as described in section 4.2.1.5.2.

4.4.1.5 Analysis of data

Analysis of data was performed as described in section 4.2.1.6.

4.4.2 Results

4.4.2.1 Clinical features

No clinically observable differences were noticed between thiamine treated and untreated groups.

4.4.2.2 Analytical data

4.4.2.2.1 Liver residue levels

The liver lead content of lead exposed animals was approximately 37 fold higher in comparison to unexposed animals. Treatment with thiamine did not have any effect on lead accumulation in the liver. There were no significant differences in liver copper, but lead exposed sheep had lower concentration than normal control sheep, and the lead exposed thiamine treated group had the lowest liver copper content. Zinc was considerably depleted ($p < 0.05$) from the liver of lead exposed sheep. Liver of these sheep contained about 50% of zinc level found in the liver of normal sheep. There was no difference between thiamine treated and untreated groups. There were no significant differences among groups with respect to liver copper, iron, and manganese. The summary of results for liver residues is presented in Table 4.4.1.

4.4.2.2.2 Kidney residue levels

The kidney of lead exposed sheep had much higher lead concentration than normal sheep. Interestingly, Pb exposed thiamine treated animals accumulated several times more lead in the kidney than lead exposed untreated animals. No significant differences among groups were noted in kidney copper, zinc, iron, and manganese. Table 4.4.2 presents a summary of kidney residue levels.

TABLE 4.4.1 The effect of repeated injections of thiamine on Pb, Cu, Zn, Fe, and Mn level in the liver of sheep chronically exposed to lead in the diet.

Variable	Normal	Pb Exposed		ANOVA P<
	control	untreated	thiamine treated	
		ug/g DM ^a		
(n)	2	2	2	
Pb	0.47 ^b ±0.15	17.02 ^c ±0.47	17.59 ^c ±0.82	0.0003
Cu	441.96±79.07	345.80±56.20	258.55±51.75	0.2707
Zn	240.67 ^c ±8.37	116.95 ^b ±6.25	125.01 ^b ±23.4	0.0162
Fe	218.62±43.34	180.40±46.01	305.15±43.45	0.2708
Mn	14.85±1.68	14.98±1.33	17.65±3.85	0.7082

n Refers to number of animals sampled per treatment.

^a Results are expressed as mean ± SE.

^{bc} Values with different superscripts within the row are significantly different (p<0.05).

TABLE 4.4.2 The effect of repeated injections of thiamine on Pb, Cu, Zn, Fe, and Mn level in the kidney (cortex) of sheep chronically exposed to lead in the diet.

Variable	Normal control	Pb Exposed		ANOVA P<
		untreated ug/g DM ^a	thiamine treated	
(n)	2	2	2	
Pb	0.99 ^b ±0.29	54.4 ^c ±3.75	204.77 ^d ±5.93	0.0001
Cu	24.24±3.05	24.85±4.45	21.55±4.75	0.0844
Zn	134.1±3.53	142.25±6.15	138.35±29.05	0.9467
Fe	167.93±16.57	268.05±52.65	199.65±33.05	0.2933
Mn	5.44±0.40	4.90±0.10	5.50±1.00	0.7743

n Refers to number of animals sampled per treatment.

^a Results are expressed as mean ± SE.

bcd Values with different superscripts within the row are significantly different (p<0.05).

4.4.2.2.3 Heart residue levels

Lead in the heart tissue of animals exposed to lead was higher than compared to unexposed animals. Treatment with thiamine did not have significant effect on heart lead content but concentration was slightly higher in the thiamine treated group. Iron content was approximately 80% higher in sheep exposed to lead in comparison to normal control, but there was no difference between thiamine treated and untreated groups. Heart copper, zinc, and manganese levels were not different between lead exposed and unexposed sheep (Table 4.4.3).

4.4.2.2.4 Spleen residue levels

Both groups of lead exposed sheep accumulated more lead in the spleen than normal sheep. The thiamine treated group accumulated significantly more lead in comparison to the lead exposed untreated group. Copper level in the spleen was also significantly higher in lead exposed groups in comparison to normal control. However, the lead exposed untreated group had significantly higher spleen copper than the thiamine treated group. Spleen zinc, iron, and manganese were not statistically different either between the lead exposed group and the normal control group, or between the two groups of lead exposed sheep. However, thiamine treated animals had higher spleen content of these metals than those animals exposed to lead but untreated. The results for spleen

TABLE 4.4.3 The effect of repeated injections of thiamine on Pb, Cu, Zn, Fe, and Mn level in the heart of sheep chronically exposed to lead in the diet.

Variable	Normal	Pb Exposed		ANOVA P<
	control	untreated ug/g DM ^a	thiamine treated	
(n)	2	2	2	
Pb	0.28 ^b ±0.01	0.86 ^c ±0.08	0.96 ^c ±0.16	0.0312
Cu	16.12±0.95	19.65±1.55	17.90±0.20	0.2065
Zn	82.61±4.41	84.83±3.08	87.50±2.40	0.6404
Fe	149.94±3.93	278.05 ^c ±9.15	259.75 ^c ±2.65	0.0011
Mn	1.69±0.24	1.55±0.05	1.48±0.43	0.8793

n Refers to number of animals sampled per treatment.

^a Results are expressed as mean ± SE.

^{bc} Values with different superscripts within the row are significantly different (p<0.05).

residue levels are summarized in Table 4.4.4.

4.4.2.2.5 Brain residue levels

Brain lead content was not significantly different among tested groups, although lead exposed sheep had higher levels of lead in their brains than normal control sheep, and thiamine treated sheep had lower levels as compared to lead exposed but untreated sheep. Animals exposed to lead had significantly higher brain iron than normal control, however, there was no difference between thiamine treated and untreated groups. Brain manganese content, on the other hand, was significantly lower in lead exposed sheep. Lead exposed untreated and thiamine treated sheep had similar brain manganese content. The summary of results for brain residue levels is shown in Table 4.4.5.

4.4.2.2.6 Residue levels of Pb, Cu, Zn, Mn, Ca, and P in the bone.

Bone lead content was significantly higher in lead exposed sheep. Thiamine treated animals had lower levels of lead in bone, however, the difference was not statistically significant at a level $P < 0.05$. Sheep exposed to lead treated with thiamine had significantly lower bone phosphorous in comparison to normal control sheep but not when compared to lead exposed untreated sheep. Also there was no significant difference between lead exposed untreated and normal sheep.

TABLE 4.4.4 The effect of repeated injections of thiamine on Pb, Cu, Zn, Fe, and Mn level in the spleen of sheep chronically exposed to lead in the diet.

Variable	Normal	Pb Exposed		ANOVA
	control	untreated	thiamine treated	P<
		ug/g DM ^a		
(n)	2	2	2	
Pb	0.13 ^b ±0.03	3.76 ^c ±0.20	18.04 ^d ±0.32	0.0001
Cu	3.49 ^b ±0.22	10.31 ^c ±0.49	5.65 ^d ±0.15	0.0014
Zn	107.47±17.59	59.66±2.49	99.65±9.15	0.1123
Fe	614.51±159.42	543.13±124.3	879.6±168.3	0.3793
Mn	0.95±0.3	0.96±0.1	2.15±0.55	0.1620

n Refers to number of animals sampled per treatment.

^a Results are expressed as mean ± SE.

bcd Values with different superscripts within the row are significantly different (p<0.05).

TABLE 4.4.5 The effect of repeated injections of thiamine on Pb, Cu, Zn, Fe, and Mn level in the brain of sheep chronically exposed to lead in the diet.

Variable	Normal control	Pb Exposed		ANOVA P<
		untreated ug/g DM ^a	thiamine treated	
(n)	2	2	2	
Pb	0.20±0.13	5.62±2.32	3.86±1.33	0.1796
Cu	13.20±1.90	17.60±1.40	19.75±1.95	0.1610
Zn	65.15±0.35	66.65±4.65	74.65±4.15	0.2796
Fe	56.2 ^b ±1.20	98.7 ^c ±10.7	104.6 ^c ±4.5	0.0265
Mn	1.68 ^c ±0.01	1.3 ^b ±0.1	1.1 ^b ±0.0	0.0129

n Refers to number of animals sampled per treatment.

^a Results are expressed as mean ± SE.

^{b,c} Values with different superscripts within the row are significantly different (p<0.05).

The bone content of copper, zinc, iron, manganese, and calcium was not statistically different among all groups. Table 4.4.6 contains a summary of results for bone.

4.4.2.2.7 Lead accumulation in the tissues and the effect of treatment with thiamine: A general overview

Figure 4.4.1 shows interrelations among different tissues with respect to lead content in normal and lead exposed untreated and thiamine treated sheep. The ability to accumulate lead varies among tissues. Liver, kidney, spleen, and brain had relatively higher lead content than heart and bone as compared to normal control levels. The highest lead content was in the kidney, followed by liver, spleen, and brain, whereas the lowest lead was in the heart. Treatment with thiamine significantly increased lead content in kidney (~3.8 times) and in spleen (~4.6 times) but did not affect levels in liver and heart. A slight decline in lead content was noted in brain (~30%) and bone (~23%) in thiamine treated animals.

4.4.3 Discussion

Bratton et al (1981a,b) found that thiamine when administered simultaneously with lead prevented accumulation of lead in liver, kidney, bone, and brain tissue in calves. Their experiment however, did not explain whether thiamine decreased absorption, increased excretion, or prevented

TABLE 4.4.6 The effect of repeated injections of thiamine on Pb, Cu, Zn, Fe, Mn, Ca, and P level in the bone of sheep chronically exposed to lead in the diet.

Variable	Normal control	Pb Exposed		ANOVA P<
		untreated ug/g or % Ash ^a	thiamine treated	
n)	2	2	2	
Pb	9.65 ^b ±7.44	94.18 ^c ±1.89	73.27 ^c ±5.34	0.0033
Cu	6.55±0.15	6.49±0.11	6.76±0.04	0.3115
Zn	188.74±7.12	148.45±10.7	131.34±18.45	0.1070
Fe	38.47±1.23	38.59±7.51	30.92±0.09	0.4644
Mn	7.36±0.18	6.89±0.19	6.89±0.09	0.1927
Ca %	42.05±0.25	40.80±1.40	37.75±1.55	0.1738
P %	4.05 ^c ±0.12	3.65 ^{bc} ±0.08	3.5 ^b ±0.07	0.0490

Refers to number of animals sampled per treatment.

^a Results are expressed as mean ± SE.

^c Values with different superscripts within the row are significantly different (p<0.05).

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deposition of lead in these organs by some other mechanism. Experiments conducted on rodents provided similar results (Flora and Sharma 1986, Flora and Tandon 1986, Kim 1988). Somewhat different results were presented by Karchru et al (1987). These authors did not find thiamine to be able to lower lead content in the liver, kidney, and brain. The findings from the present study are in accordance with those observed on calves (Bratton et al 1981a,b) with respect to brain and bone tissue, but there is no consensus about liver and kidney. Liver lead content was not affected by thiamine treatment and kidney lead was significantly elevated. Lack of change in liver lead content can be explained by differences in species, age, diet, route of administration of thiamine, or other factors. However, metabolic significance of enhanced accumulation of lead in the kidney after treatment with thiamine remains obscure. Previous experiments have shown that thiamine did increase urinary lead excretion (see sections 4.2 and 4.3). Hence the higher content of lead in the kidney can be an effect of tubular reabsorption of lead filtered at a glomerulus. Large fraction of glomerular ultrafiltrate is reabsorbed (Vander et al 1977). Some evidence indicates that renal excretion has a maximum limit above which reabsorption and hence retention increases (Goyer 1971). It is not known whether thiamine induced increased level of lead in the sheep's kidney can cause functional impairment or lead is only transiently

deposited in harmless form. However, the fact that thiamine treatment causes accumulation of lead in the kidney cannot be ignored, since it can cause lead nephropathy. Another intriguing observation in this study was the accumulation of lead in the spleen after injections of thiamine. Kim (1988) reported that thiamine 50 mg/kg increased spleen lead when lead was administered intraperitoneally and decreased when lead was administered orally. Increased osmotic fragility and other structural changes of RBC resulting from the effect of lead on erythrocytes membrane (Horiguchi 1984, Fukumoto et al 1983), depressions of Na^+/K^+ ATPase (Secchi et al 1973), decrease in the reduced glutathione content of RBC (Howard 1978) can cause decreased erythrocytes survival time as well as functional impairment. Considering the physiological role of the spleen as "graveyard for erythrocytes" the action of thiamine can explain the increased level of lead in the spleen. Thiamine apparently has the ability (proposed mechanism will be discussed in General discussion) to mobilize lead from its intracellular storage site (evidence is increased blood level after thiamine injection). It is probable that a massive release of sequestered lead to the blood could cause extensive damage to RBC and injured erythrocytes would be eliminated by the spleen from peripheral circulation.

There is a vast body of literature about the effects of essential elements on lead metabolites. The effects of

copper, zinc, iron, calcium, and phosphorus are well investigated. However, considerably less information has been gathered concerning the effects of lead on the metabolism of essential metals. The present study provided basic information about the effects of lead on residue levels of copper, zinc, iron, and manganese in some tissues of sheep with chronic lead poisoning. Lead is known to compete with zinc and can even replace zinc. Both groups of lead exposed sheep had significantly lower liver zinc in comparison to normal control. There are relatively few reports referring to the effects of lead on function of the liver. The metabolic implications of lowered zinc in the liver are not known.

Copper content was significantly higher in the spleens of lead exposed sheep, and the untreated group had a significantly higher level than the thiamine treated group. It is interesting to note that the distribution of copper content in the spleen between lead exposed untreated and thiamine treated groups was exactly opposite to distribution of lead where the untreated group had significantly lower lead content in the spleen as compared to the thiamine treated group.

Higher iron content of brain and heart tissues in lead exposed sheep as compared to normal control sheep apparently reflects the toxic effects of lead on heme synthesis. Lead interferes with utilization of iron for heme formation in the

mitochondria causing excessive accumulation of iron in the mitochondria (Sassa 1978). Brain and heart have an extremely high demand for energy, and heme iron is a vital part of several enzymes of the electron transport pathway and other heme proteins. Apparently a compromised feedback inhibition mechanism caused excessive accumulation of iron.

Brain manganese was significantly lower in sheep exposed to lead. The effect of lead on manganese metabolism is not known. It is not known whether observed lower level of manganese could have any impact on functional activity of manganese dependent enzymes in the brain, since no reference values regarding deficiency threshold of this metal in the brain are available.

Phosphorus level in the bone of the lead exposed thiamine treated group was lower than in the remaining two groups, although the difference with lead exposed untreated group was not significant. Lead is known to be able to displace calcium in the bone, and is deposited as lead phosphate (Barry and Mossman 1970). The metabolism of lead in bone appears to be dependent on multiple interactions of hormones and other factors integrating bone activity, and regulated by dynamic balance between bone formation and bone resorption. Osteoclasts have been found to play a central role in the metabolism of lead in the bone (Eisenstein and Kawonoue 1975, Hsu et al 1973, VanMullen and Stadhouders 1974). Bone lead metabolism is probably integrated very tightly with calcium

and phosphorus metabolism. Pound and Mittelstaedt (1983) have shown that certain physiological stimuli can mobilize lead and calcium in cultured hepatocytes. The direct action of thiamine on bone lead metabolism is not known, however, one would expect that removal of lead from bone due to action of thiamine would be followed by removal of phosphorus as well, whereas the relative level of calcium would remain unchanged.

**Publications and Information Arising from the ADF Funded Mineral Research
Program 1982-1988**

(A) PUBLICATIONS from research carried out at Department of Animal and Poultry Science, University of Saskatchewan, Saskatoon between Nov. 1982 and Oct. 1988 are outlined below:

SUMMARY

(i) FULL PAPERS IN REFEREED JOURNALS:

(a) Published, accepted or submitted:	14
(b) In preparation (final stages): 10	17
(c) In preparation (data analysis completed):	<u>7</u>
	38

(ii) CHAPTERS IN BOOKS:

1

(iii) PAPERS IN PUBLISHED CONFERENCE PROCEEDINGS:

14

(iv) TECHNICAL REPORTS:

5

Total number of publications from research at U of S.

58

(B) OTHER ACADEMIC ACTIVITIES:

(i) INVITATIONS FOR LECTURES OUTSIDE U OF S:

6

(ii) THESIS SUPERVISED: M.Sc.

1

PUBLICATIONS

PAPERS IN REFEREED JOURNALS (accepted / submitted): (14)

- (1) S.R. Gooneratne, R. Chaplin, A.M. Trent and D.A. Christensen. (1988).
"Copper, zinc, iron and molybdenum in sheep bile; Effect of thiomolybdate administration"
British Veterinary Journal 144: 000-000.
- (2) S.R. Gooneratne, B. Laarveld, R.K. Chaplin, and D.A. Christensen. (1989).
"Profiles of ^{67}Cu and ^{99}Mo in blood, bile, urine and feces from ^{67}Cu primed lambs: Effect of tetrathiomolybdate on the metabolism of recently stored tissue copper"
British Journal of Nutrition (in the press).
- (3) S.R. Gooneratne, B. Laarveld, R.K. Chaplin, and D.A. Christensen. (1989).
"Profiles of ^{67}Cu and ^{99}Mo profiles in blood, bile, urine and feces from ^{67}Cu primed lambs: Effect of ^{99}Mo labelled tetrathiomolybdate on the metabolism of long-term storage copper"
British Journal of Nutrition (in the press)
- (4) S.R. Gooneratne and D.A. Christensen. (1989, March issue).
"A survey of maternal copper status and fetal tissue copper concentration in Saskatchewan bovine"
Canadian Journal of Animal Science (in the press)
- (5) S.R. Gooneratne and D.A. Christensen. (1989).
"A survey of maternal-fetal tissue zinc, iron, manganese and selenium concentration in bovine"
Canadian Journal of Animal Science (in the press)
- (6) S.R. Gooneratne, A. Olkowski, R.G. Klemmer, G.A. Kessler and D.A. Christensen. (1989).
"High sulfur related thiamine deficiency in cattle; A field study"
Canadian Veterinary Journal (in the press)
- (7) R. Gooneratne, B. Laarveld and D.A. Christensen. (1989).
"Effect of ^{67}Cu and ^{99}Mo labelled tetrathiomolybdate on the distribution of ^{67}Cu , Cu and ^{99}Mo in bile fractions in sheep"
Journal of Inorganic Biochemistry (in the press)
- (8) S.R. Gooneratne, D.A. Christensen, and B.S. Wenger. (1989).
"Effects of thiomolybdates in chick embryos"
Nutrition Reports International (submitted)
- (9) S.R. Gooneratne, A.A. Olkowski and D.A. Christensen. (1989).
"Sulfur induced polioencephalomalacia in cattle: Some biochemical changes"
Canadian Journal of Veterinary Research (submitted)

- (10) S.R. Gooneratne, H.W. Symonds, J.V. Bailey and D.A. Christensen. (1989).
"Effect of dietary Cu, Mo, S levels on biliary Cu and Zn excretion in Simmental and Angus cattle"
British Journal of Nutrition (submitted)
- (11) S.R. Gooneratne, W.R. Buckley and D.A. Christensen. (1989).
"Copper metabolism and deficiency in ruminants. Influence of molybdenum and sulfur and stable isotope studies"
Canadian Journal of Animal Science (in the press)
- (12) A.A. Olkowski, S.R. Gooneratne, E.C. Crichlow, C.G. Rousseaux and D.A. Christensen. (1989).
"Effects of high dietary sulfur on brain functions using evoked potentials technique"
Canadian Journal of Veterinary Research (submitted)
- (13) A.A. Olkowski, S.R. Gooneratne and D.A. Christensen. (1989).
"Effects of high dietary sulfur and different concentrations of Cu, Mo and thiamine on in-vitro phagocytic and candidacidal activity of neutrophils in ovine"
Canadian Journal of Veterinary Research (submitted)
- (14) A.A. Olkowski and S.R. Gooneratne. (1989).
"Microbiological methods of thiamine measurement in biological material using 96-well microplate reader"
Veterinary Research Communications (submitted)

PAPERS IN REFEREED JOURNALS (in preparation): (23)

- (1) S.R. Gooneratne, T. Berryere and D.A. Christensen.
"Effect of dietary Cu, Mo, S levels and tetrathiomolybdate administration on urine excretion of Cu and Zn in Simmental and Angus cattle"
Research in Veterinary Science
- (2) S.R. Gooneratne, M.E. Smart, B. Laarveld, J.G. Ferguson, J.V. Bailey and D.A. Christensen.
"Effects of high S induced changes in maternal-fetal liver copper status in bovine"
Canadian Journal of Animal Science
- (3) S.R. Gooneratne, T. Berryere, M.E. Smart, B. Laarveld and D.A. Christensen.
"Intracellular distribution of copper in liver during sulfur, and copper supplementation in bovine"
Canadian Journal of Animal Science
- (4) S.R. Gooneratne, M.E. Smart, W.E. Howell and D.A. Christensen.
"Effect of Cu supplementation on carcass characteristics and liver and kidney Cu, Zn and Fe concentrations in male and female lambs"
Research in Veterinary Science

- (5) S.R. Gooneratne, M.E. Smart, W.E. Howell and D.A. Christensen.
"A comparison of blood Cu, Zn, Fe concentrations and different methods of extraction of plasma Cu, Zn and Fe in male and female lambs"
Research in Veterinary Science
- (6) S.R. Gooneratne and D.A. Christensen.
Influence of chelating agents in excretion of Cu, Zn and Fe in sheep bile and urine"
Toxicology Letters
- (7) S.R. Gooneratne and D.A. Christensen.
"Effect of varied doses of tetrathiomolybdate on bile Cu excretion in Simmental and Angus cattle"
British Veterinary Journal
- (8) S.R. Gooneratne and D.A. Christensen.
"Changes in bile flow and bile Cu, Zn concentration during pregnancy in Simmental and Angus cattle"
Research in Veterinary Science
- (9) S.R. Gooneratne, B. Laarveld and D.A. Christensen.
"Enterohepatic circulation of ^{67}Cu and ^{99}Mo in sheep"
Veterinary Research Communications
- (10) S.R. Gooneratne, B. Laarveld and D.A. Christensen.
"Subcellular distribution of ^{67}Cu and ^{99}Mo in the liver and kidney of sheep following an infusion of ^{99}Mo labelled tetrathiomolybdate given intravenously 24 hours after an intravenous ^{67}Cu injection"
Canadian Journal of Veterinary Research
- (11) S.R. Gooneratne and D.A. Christensen.
"Role of thiol in biliary Cu excretion in cattle"
Veterinary Research Communications
- (12) S.R. Gooneratne and D.A. Christensen.
"A comparison of bile and urine Cu excretion in sheep, cattle and pigs following intravenous tetrathiomolybdate administration"
Veterinary Research Communications
- (13) S.R. Gooneratne, J.G. Gagnon and D.A. Christensen.
"Effect of varied concentrations of Cu, S, and thiamine on blood Cu, Zn, Fe and thiamine concentrations in the rat"
Nutrition Research
- (14) S.R. Gooneratne, J.G. Gagnon and D.A. Christensen.
"Effect of different concentrations of Cu, S and thiamine on liver, kidney, heart, spleen, Cu, Zn, Fe and thiamine concentration in the rat"
Nutrition Research

- (15) S.R. Gooneratne, A.A. Olkowski and D.A. Christensen.
"Changes in plasma Cu, ceruloplasmin and thiamine concentration in calf during first 4 weeks after birth"
Veterinary Research Communications
- (16) S.R. Gooneratne, S. Goubau, A. Gonzalez and B.D. Murphy.
"Profiles of plasma Cu changes during immunization of sheep against GnRH; Effect of GnRH carrier"
Canadian Journal of Veterinary Research
- (17) S.R. Gooneratne, B.P. Goplen, R. Muir, M. Mostrom, J.M. Bell and D.A. Christensen.
"Pharmacodynamics and pharmacokinetics of orally administered 3,3 methylene bishydroxycoumarin in cattle"
American Journal of Veterinary Research
- (18) A. Olkowski, S.R. Gooneratne, J.V. Bailey and D.A. Christensen
"Effect of differing levels of Cu in high S diets in thiamine metabolism in sheep"
Journal of Agricultural Science, Cambridge
- (19) A. Olkowski, S.R. Gooneratne, J.V. Bailey and D.A. Christensen.
"Effects of sulfur and thiamine on copper metabolism in sheep"
British Journal of Nutrition
- (20) A. Olkowski, S.R. Gooneratne and D.A. Christensen.
"Effect of varied doses of thiamine on excretion of lead in bile, urine and faeces in lead-loaded sheep"
Research in Veterinary Science
- (21) A.A. Olkowski, S.R. Gooneratne and D.A. Christensen.
"Comparison of changes in lead and thiamine levels in blood, bile, urine and faeces in lead-loaded sheep treated with thiamine and/or EDTA"
Journal of Veterinary and Human Toxicology
- (22) A.A. Olkowski, S.R. Gooneratne and D.A. Christensen.
"Potential use of thiamine in the treatment of lead toxicity in cattle"
American Journal of Veterinary Research
- (23) A.A. Olkowski, C.G.R. Rousseaux, A. Chauvet, S.R. Gooneratne and D.A. Christensen.
"Pathology of high sulfur induced encephalopathy in sheep"
Journal of Comparative Pathology
- (24) R. Muir, B.P. Goplen, S.R. Gooneratne, M. Mostrom, J.M. Bell and D.A. Christensen.
"A HPLC method for assay of dicoumarol concentration in body fluids of cattle"
Journal of High Performance Liquid Chromatography

(11) CHAPTERS IN BOOKS: (1)

- (1) J. McC. Howell and S.R. Gooneratne. (1987).
 "The pathology of copper toxicity in animals".
 In "Copper in Animals and Man", pp 53-78, Eds. J.McC. Howell and J.M.
 Gawthorne. CRC Press Inc. Florida, 1987.

(111) PAPERS IN PUBLISHED CONFERENCE PROCEEDINGS: (14)

- (1) S.R. Gooneratne, W.E. Howell, D.A. Christensen and M.E. Smart.
 "Comparison of hepatic copper retention by male and female lambs".
 In the Proceedings of the Western branch meeting of the Canadian Society
 of Animal Science, Saskatoon, p. 15, June 1983
- (2) S.R. Gooneratne and D.A. Christensen.
 "Increased copper excretion in bile after thiomolybdate administration".
Federation Proceedings 43, 790, (1984).
- (3) S.R. Gooneratne, B. Laarveld, D.A. Christensen and R.K. Chaplin.
 "Biliary copper excretion and the effect of route of administration of
 tetrathiomolybdate on liver copper removal in sheep".
Journal of Animal Science, suppl. 1, 504, (1985).
- (4) S.R. Gooneratne and D.A. Christensen.
 "Gestation age on maternal-fetal liver copper levels in bovine".
 In the Proceedings of the 5th International Symposium on Trace Element
 Metabolism in Man and Animals, 334-337, (1985). Eds. C.F. Mills, I.
 Bremner and C.K. Chesters. Commonwealth Agriculture Bureau, Scotland.
- (5) S.R. Gooneratne, D.A. Christensen, R. Chaplin and A.M. Trent.
 "Copper: Biliary secretion in sheep and the effects of thiomolybdate
 administration".
 In the Proceedings of the 5th International Symposium on Trace Element
 Metabolism in Man and Animals, 342-346, (1985). Eds. C.F. Mills, I.
 Bremner and C.K. Chesters. Commonwealth Agriculture Bureau, Scotland.
- (6) S.R. Gooneratne, B. Laarveld and D.A. Christensen.
 "Effect of tetrathiomolybdate on the distribution of ^{67}Cu and ^{99}Mo in
 bile fractions".
Canadian Journal of Animal Science, 66, 1198 (1986)
- (7) S.R. Gooneratne, B. Laarveld, M.E. Smart, J.G. Ferguson, J.V.
 Bailey and D.A. Christensen.
 "Changes in maternal-fetal liver copper levels in bovine following copper
 and sulfur supplementation".
Canadian Journal of Animal Science, 66, 1198-1199 (1986)

- (8) S.R. Gooneratne.
 "Potential use of thiomolybdate in the treatment of copper overload diseases".
Acta Pharmacologica et Toxicologica, 59, suppl. 7, 518-523 (1986).
- (9) S.R. Gooneratne.
 "Copper nutrition of beef cattle; Effect of sulfate and molybdenum and species susceptibility".
 In the Proceedings of the 7th Western Nutrition Conference, p. 266-292.
 University of Saskatchewan, Saskatoon, (1986).
- (10) S.R. Gooneratne, D.A. Christensen, J.V. Bailey and H.W. Symonds.
 "Influence of breed, and dietary Cu, Mo and S levels on biliary Cu excretion in cattle".
 In the Proceedings of the 6th International Symposium on Trace Element Metabolism in Man and Animals, p. 52. Asilomar Conference Centre, Pacific Grove, California, June 1987.
- (11) S.R. Gooneratne, B. Laarveld and D.A. Christensen.
 "Tetrathiomolybdate in the treatment of copper storage diseases".
 In Toxicology of metals: Clinical and Experimental Research, p. 321-322, Eds. S. Brown and Y. Kodama. Ellis Horwood Ltd, 1987.
- (12) S.R. Gooneratne, B. Laarveld, M.E. Smart, J.G. Ferguson, J.V. Bailey and D.A. Christensen.
 Effect of Cu and S supplementation of pregnant cows on subcellular distribution of Cu in fetal/calf liver pre- and post-partum".
Canadian Journal of Animal Science, 67, 1187-1188 (1987)
- (13) S.R. Gooneratne, J.V. Bailey, H.W. Symonds and D.A. Christensen.
 "Effect of dietary Cu, Mo and S levels on bile and urine Cu excretion in 2 breeds of cattle".
Canadian Journal of Animal Science, 67, 1188 (1987)
- (14) S.R. Gooneratne. (1987).
 "Copper, molybdenum, sulfate nutrition in ruminants"
 In the Proceedings of the Symposium on "Abomasitis and Abomasal Ulcers in Ruminants", University of Wyoming, Laramie, Wyoming Eds. F.C. Hinds and L.F. Woodard, pp 12-41.
- (15) A.A. Olkowski, C.G. Rousseaux, A. Chauvet, S.R. Gooneratne and D.A. Christensen. (1988).
 "Polioencephalomalacia in sheep associated with high dietary sulphur intake"
 In the Proceedings of the Annual Meeting of the American College of Veterinary Pathology

(iv) TECHNICAL REPORTS: (5)

- (1) S.R. Gooneratne, D.A. Christensen, B. Laarveld and M. Smart.
"Copper deficiency studies in cattle"
College of Agriculture Highlights, University of Saskatchewan, Saskatoon, Saskatchewan, p. 16, (1985).
- (2) S.R. Gooneratne, B. Laarveld and D.A. Christensen.
"Tetrathiomolybdate in the treatment of copper storage diseases".
Department of Animal and Poultry Science Research Report, University of Saskatchewan, Saskatoon, Saskatchewan, p. 97-101, (1986).
- (3) S.R. Gooneratne, D.A. Christensen, B. Laarveld and M.E. Smart.
"Maternal-Fetal copper metabolism in Cattle".
In Termuende Research Station Report, p. 19-20. Ed. R.D.H. Cohen and B.D. King, University of Saskatchewan, Saskatoon, June, 1986.
- (4) S.R. Gooneratne, M.E. Smart, B. Laarveld, J.G. Ferguson, J.V. Bailey, and D.A. Christensen.
"Maternal - fetal effects during copper and sulfur supplementation".
In the Department of Animal and Poultry Science Research Report, pp 18-23, University of Saskatchewan, Saskatoon. 1987.
- (5) S.R. Gooneratne and D.A. Christensen.
"Survey of copper status of cattle in Saskatchewan and influence of gestation age and maternal-fetal liver copper levels in bovine".
In the Department of Animal and Poultry Science Research Report, pp 31-35, University of Saskatchewan, Saskatoon. 1987.

OTHER ACADEMIC ACTIVITIES(1) INVITATIONS FOR LECTURES OUTSIDE U. OF S.: (6)

- (1) S.R. Gooneratne (1988).
Invited to present a lecture on "Thiomolybdate chelation therapy in copper toxicity in sheep" at the Atomic Energy Canada Ltd, Chalkriver, Ontario, Canada, in February.
- (2) S.R. Gooneratne (1987).
Invited to present a plenary lecture on "Copper, molybdenum, sulfate nutrition in ruminants" at a conference on Abomasitis/Abomasal Ulcers of the young ruminant (sponsored by Departments of Veterinary Sciences and Animal Science, University of Wyoming), Fosters Convention Centre, Laramie, Wyoming, USA, in September.
- (3) S.R. Gooneratne (1987).
Invited to present a lecture on "Copper/Molybdenum interaction in ruminants" at the Department of Animal and Poultry Science, University of British Columbia, Vancouver, Canada, in March.

- (4) S.R. Gooneratne (1986).
Invited to present a lecture on "Copper nutrition of beef cattle; Effects of sulfate and molybdenum and species susceptibility" at the 7th Western Nutrition Conference, Saskatoon, Canada, in September.
 - (5) S.R. Gooneratne (1985).
Invited to present a lecture on "Thiomolybdate and its use in copper storage diseases" at The Department of Veterinary Pathology, University of Utrecht, The Netherlands, in June.
 - (6) S.R. Gooneratne (1985).
Invited to present a plenary lecture on 'Trace element toxicity problems in animals' at the Symposium on Animal Health Disturbances Related to Unbalanced Trace Element Nutrition, The National Veterinary Institute, Uppsala, Sweden, in June.
 - (7) S.R. Gooneratne (1985).
Invited to present a lecture on "Pathology and biochemistry of copper toxicity in sheep" at The National Veterinary Institute, Oslo, Norway, in June.
- (11) THESIS SUPERVISED: (1)
- "Effect of thiamine on lead excretion in ruminants" - A.A. Olkowski, M.Sc. (1988)

UNIVERSITY OF SASKATCHEWAN
Department of Agriculture Project No. 2.2-17
"Interactions of Thiamin, Copper, Sulfur,
& Lead in Ruminants"
Under the Direction of D.A. Christensen, Animal & Poultry Science
Statement of Receipts & Expenditures for the Period
May 19 - November 30, 1988

Receipts

Government of Saskatchewan - June, 1988 \$ 15,000

Expenditures

Salaries	\$22,358	
Fringe Benefits	2,130	
Materials, Supplies	6,246	
Printing	49	
Telephone	217	
	<u> </u>	<u>31,000</u>

Oyerexpenditure as of November 30, 1988 \$(16,000)


PROJECT DIRECTOR


W. E. SMITH
RESEARCH ACCOUNTANT

COPY FOR YOUR RECORDS

List of people and equipment

Dr. R.S. Gooneratne	-	Research scientist	100%
T. Berryere	-	Technician	80%
L.S. Manchester	-	Summer student	25%
A. Olkowski	-	Graduate student	50%
A. Moosa	-	Student assistant	5%
A.F. Olender	-	Student assistant	5%
B. Goldade	-	Graduate student	5%

No equipment purchased over \$500.00.